Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 858 (2007) 96–105
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Liquid chromatography-electrospray ionization-tandem mass spectrometry for simultaneous analysis of chlorogenic acids and their metabolites in human plasma

Yuji Matsui, Shun Nakamura, Naoki Kondou, Yoshio Takasu, Ryuji Ochiai, Yoshinori Masukawa *

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

Received 1 February 2007; accepted 12 August 2007
Available online 19 August 2007

Abstract

A method using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) was developed for the simultaneous analysis of nine chlorogenic acids (CGAs), three isomers each of caffeoylquinic acids (CQAs), feruloylquinic acids (FQAs) and dicaffeoylquinic acids (dCQAs), and their two metabolites, caffeic acid (CA) and ferulic acid (FA), in human plasma. In simultaneous multiple reaction monitoring (MRM) measurements using ESI-MS/MS with a negative ion mode, a deprotonated molecular ion derived from each of the 11 molecules was used as a precursor ion while three diagnostic product ions characteristic for each were selected for the qualitative analysis. To obtain maximal intensities for all diagnostic product ions, the collision energy was optimized for each one. LC separation was achieved under conditions of a reversed-phase Inertsil ODS-2 column combined with a gradient elution system using 50 mM acetic acid with 3% acetonitrile aqueous solution and 50 mM acetic acid with 100% acetonitrile. In the quantitative analysis, one of the three diagnostic product ions for each of the 11 molecules was selected. Application of simultaneous LC-ESI-MS/MS MRM measurements to analyze the 11 standards spiked into blank human plasma indicated that all diagnostic product ions were detected without any interference, and that the sensitivity, linearity and recovery of this method were acceptable. When using this method to analyze those 11 molecules in the plasma after oral ingestion of 250 ml of a drink containing a green coffee bean extract (300 mg CGAs), all 11 molecules were identified and CQAs, FQAs and FA were quantified. CQAs, FQAs and dCQAs in human plasma were detected for the first time. This method should be useful to understand the biological and pharmacological effects of CGAs, such as improvement of human hypertension.

Keywords: Chlorogenic acids; Human plasma; LC-ESI-MS/MS; Metabolite; Simultaneous analysis

1. Introduction

Chlorogenic acids (CGAs), a family of esters of quinic acid (QA) and caffeic acid (CA) or ferulic acid (FA), have been reported to have diverse biological and pharmacological effects, such as antioxidant activities [1], anti-carcinogenic activities [2] and antihypertensive activities [3,4]. Among them, attention has been paid to the effect of CGAs on improving human hypertension [3,4], because the incidence of hypertensive diseases (so-called lifestyle-related diseases) has been recently increasing in many countries all over the world. Humans routinely ingest CGAs from various plant foods including vegetables [5] and fruits [6]. Since coffee beans contain high levels of CGAs [7], humans also get CGAs from coffee drinks (infusions of coffee beans) that are widely consumed worldwide and from coffee-containing beverages. The CGAs in green coffee beans consist mainly of three isomers each of caffeoylquinic acids (3-, 4- and 5-CQAs), feruloylquinic acids (3-, 4- and 5-FQAs) and dicaffeoylquinic acids (3,4-, 4,5- and 3,5-dCQAs) [7,8], as shown in Fig. 1. Those CGAs are also found in roasted coffee beans. It has been reported that two metabolites, CA and FA, are detected in rat plasma and urine after oral ingestion of 5-CQA, probably due to hydrolysis and methylation in the rats [9]. However, little is known about the absorption and metabolism of the

* Corresponding author at: Analytical Research Center, Kao Corporation, 2606 Akabane, Ichikai, Haga, Tochigi 321-3497, Japan. Tel.: +81 285 68 7416; fax: +81 285 68 7418.
E-mail address: masukawa.yoshinori@kao.co.jp (Y. Masukawa).

1570-0232/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2007.08.013

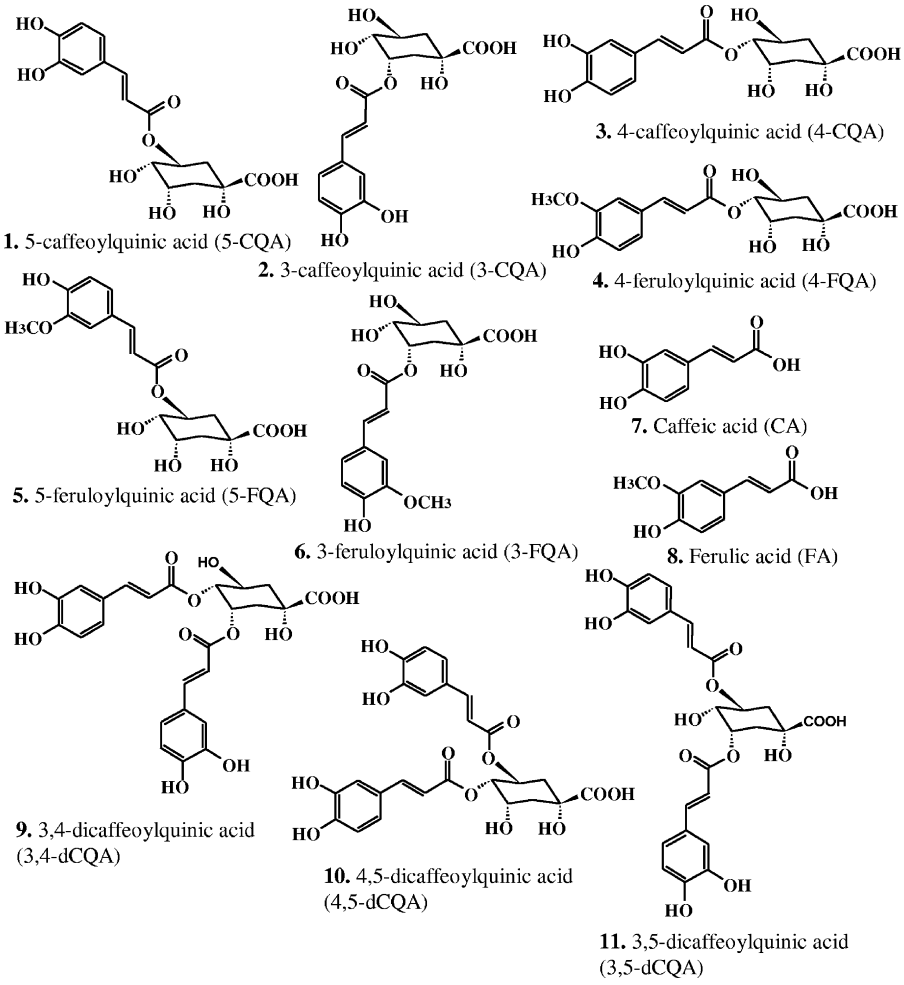

Fig. 1. Chemical structures of nine CGAs (CQAs, FQAs and dCQAs) and their metabolites (CA and FA).

nine CGAs and their two metabolic molecules, in human plasma after consumption of coffee-related drinks.

In human plasma after oral ingestion of foods containing CGAs, only CA, FA and their glucuronate/sulfate conjugates have been detected [10–12]. This indicates that CA and FA derived from CGAs are absorbed into human blood after the ingestion. However, since no method exists to detect CGAs (three isomers each of CQAs, FQAs and dCQAs) in plasma, it remains unknown whether CGAs are directly absorbed into the blood and then metabolized to CA and FA, or whether CA and FA are directly absorbed into the blood after their metabolic conversion prior to the absorption. On the other hand, there are reports that low but significant levels of CQAs can be detected in human urine after oral ingestion of CQAs [10,13,14]. This suggests that CGAs ingested orally may be directly absorbed into human blood, followed by the excretion of intact CGAs in the urine. To understand the pharmacokinetics of CGAs and their beneficial effects on human hypertension, all nine CGAs and their two metabolites (CA and FA) need to be analyzed in human plasma. In particular, as the first step, the biological phenomenon about whether CGAs are directly absorbed into human blood needs to be clarified.

Methods using LC coupled to electrochemical detection (LC-ECD) [11,12] and to ultraviolet detection (LC-UVD) [13,15,16] have been applied for the qualitative and quantitative analysis of CA, FA and their conjugates in human/animal plasma and urine after the ingestion of CGAs. However, those methods are too insensitive to detect the extremely low levels of CGAs which may be present in human plasma. Further, there is insufficient specificity in the detection since the qualitative information depends only on retention times of detected peaks in the LC. There is another method that uses GC–MS to analyze 5-CQA in urine [14]. However, that method seems to be too insensitive to detect a lower level of 5-CQA which is expected to be present in human plasma, based on the description regarding the limit of detection (LOD) for 5-CQA in urine being as high as 4 mg/l [14]. Recently, sensitive and specific methods for the analysis of CGAs using LC–MS or LC–MS/MS have been reported and then applied to the qualification and quantification of 5-CGA, CA and FA in human urine [10] and in rat plasma and urine [17,18]. However, there were no methods that could be reliably applied to the simultaneous analysis of all 11 molecules (nine CGAs and their two metabolites) in human plasma, where their levels may be extremely low, after oral ingestion of a coffee-related drink containing CGAs.

The aim of this study was to establish a method for the simultaneous analysis of all 11 CGA-related molecules in human plasma. For qualitative analysis, we used three diagnostic

product ions derived from each of the 11 molecules by simultaneous multiple reaction monitoring (MRM) measurements in LC coupled to electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). In addition, for quantitative analysis, we used one of the three diagnostic product ions with an almost maximal intensity for each of the eleven molecules using the same conditions of LC-ESI-MS/MS as the qualitative analysis. This method was used for simultaneous analysis in human plasma after oral ingestion of a drink containing a green coffee bean extract (300 mg CGAs). We succeeded in identifying CQAs, FQAs and dCQAs and in quantifying concentrations of CQAs and FQAs in human plasma for the first time.

2. Experimental

2.1. Chemicals

Standards, such as 5-CQA, CA and FA, were purchased from Sigma (St. Louis, MO, USA). Other standards, 3-CQA (purity > 98% by LC-UVD and ^{1}H-NMR), 4-CQA (>98%), 3-FQA (>97%), 4-FQA (>96%), 5-FQA (>97%), 3,4-dCQA (>96%), 4,5-dCQA (>96%) and 3,5-dCQA (>96%), were obtained by repeated fractionations of a green coffee bean extract (Hasegawa perfume, Tokyo, Japan) using preparative chromatography with an ODS column. 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 250 ml drink containing a green coffee bean extract was orally ingested in this study. That drink contained a total of 300 mg CGAs (1.20 mg/ml), consisting of 57.5 mg 3-CQA (0.23 mg/ml), 60 mg 4-CQA (0.24 mg/ml), 105.0 mg 5-CQA (0.42 mg/ml), 12.5 mg 3-FQA (0.05 mg/ml), 12.5 mg 4-FQA (0.05 mg/ml), 25.0 mg 5-FQA (0.10 mg/ml), 12.5 mg 3,4-dCQA (0.05 mg/ml), 5.0 mg 4,5-dCQA (0.02 mg/ml) and 10.0 mg 3,5-dCQA (0.04 mg/ml), as determined by quantitative LC-UVD at 325 nm.

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

2.2. Apparatus and analytical conditions

A triple quadrupole API-4000 was equipped with an ion spray (ESI) source and analyst version 1.0 software (Applied Biosystems, Foster City, CA, USA), and was interfaced with an Agilent 1100 liquid chromatograph, an 1100 well plate autosampler (Agilent Technologies, Palo Alto, CA, USA) and an Inertsil ODS-2 2.1 mm i.d. × 150 mm, particle size 5 μm column (GL Sciences, Tokyo, Japan). For flow injection-MS/MS analysis, the column was uncoupled from the system. In this LC-ESI-MS/MS system, an 1100 binary pump was connected to a carrier or two mobile phases (A, 50 mM aqueous acetic acid containing 3 vol% acetonitrile; B, 50 mM acetic acid in 100% acetonitrile) that were eluted at a flow rate of 0.2 ml/min. The mobile phases A and B were used in the LC gradient elution system based on the gradient program shown in Table 1 (a total run time of 60 min). The injection volume was 5 μl of each sample. The column temperature was maintained at 35 °C. The negative ion ESI-MS/MS conditions were as follows: ion spray voltage, −4500 V; ion source heater temperature, 600 °C; ion source gas (N_2) for nebulizing, 50 psi; ion source gas (N_2) for drying solvent, 80 psi; curtain gas (N_2), 20 psi; declustering potential, −150 V; collision gas (N_2), 3×10^{-5} torr; collision exit energy, −10 V. The scan measurement in flow injection-ESI-MS/MS analysis with negative ion ESI was employed based on the detection of precursor ions $[M-H]^-$ derived from CQAs, FQAs, dCQAs, CA and FA in Q_1. This was followed by collision-induced dissociation in Q_2 using a collision energy of 10–70 V and then followed by the detection of product ion spectra in Q_3 with a scan range *m/z* 50–400 at a scan rate 200 amu/s. The simultaneous MRM measurements for the qualitative analysis in LC-ESI-MS/MS were employed using the precursor ions $[M-H]^-$ and their three diagnostic product ions. The collision energy was 40 V for *m/z* of 353 → 135 of CQAs, 30 for *m/z* 353 → 179 and 353 → 191 of CQAs, 20 V for *m/z* 367 → 173 and 367 → 191 of FQAs, 30 V for *m/z* of 367 → 193 of FQAs, 40 V for *m/z* of 515 → 179 of dCQAs, 50 V for *m/z* of 515 → 191 of dCQAs, 30 V for *m/z* of 515 → 353 of dCQAs, 40 V for *m/z* of 179 → 89 of CA, 30 V for *m/z* of 179 → 107 of CA, 20 V for *m/z* of 179 → 135 of CA, 20 V for *m/z* of 193 → 135, 193 → 149 and 193 → 179 of FA. In contrast, the quantitative analysis was performed in the simultaneous MRM measurement using *m/z* 353 → 191 for CQAs, 367 → 193 for 3-FQA, 367 → 191 for 5-FQA, 367 → 173 for 4-FQA, 515 → 353 for dCQAs, 179 → 135 for CA, and 193 → 135 for FA. Dwell time was set at 500 ms for each product ion.

Table 1
Gradient program of the LC

Time (min)	Mobile phase A (%)[a]	Mobile phase B (%)[b]
0.0	100	0
15.0	80	20
25.0	70	30
32.0	50	50
35.0	0	100
37.0	0	100
37.1	100	0
60.0	100	0

[a] 50 mM aqueous acetic acid containing 3 vol% acetonitrile.
[b] 50 mM acetic acid in 100% acetonitrile.

2.3. Human plasma

This protocol was approved by the Ethical Committee of the Kao Corporation of Japan, based on the Recommendations from the Declaration of Helsinki. Plasma samples were obtained from five healthy nonsmoking males with normal blood pressure (#1, 30 years old; #2, 31 years old; #3, 34 years old; #4, 39 years old, #5, 42 years old), who had been prohibited from consuming cof-

fee and coffee-related beverages/foods for 3 days prior to this experiment. After fasting for 14 h, 250 ml of a drink containing a green coffee bean extract (total 300 mg CGAs) was orally ingested and no other drinks were taken until collection of blood had been completed. Blood samples were taken using heparin as an anticoagulant before and 1 and 3 h after the ingestion. They were immediately centrifuged at 4,000 × *g* for 10 min at 5 °C to prepare plasma. 2.5 ml of plasma was mixed with 50 μl 0.4 M phosphate buffer (pH 3.6) containing 20 wt.% ascorbic acid and 0.1 wt.% disodium ethylenediaminetetraacetate in a polypropylene tube and was instantly subjected to the sample preparation procedure described below. The plasma obtained from subject #1 before the ingestion was used as blank plasma in this study.

2.4. Sample preparation

Preparative procedures were performed according to our previous report [19]. Thus, to 250 μl of plasma thawed just prior to use, 25 μl 6 M perchloric acid and 125 μl acetonitrile were added. The mixtures were vigorously mixed for 2 min with a vortex mixer in a polypropylene tube. Each mixture was then kept at 5 °C for 30 min, and was then centrifuged at 20,000 × *g* for 5 min at 5 °C (Eppendorf 5417R, Hamburg, Germany). The supernatants and 100 μl 0.75 M potassium carbonate solution were combined with moderate mixing for 30 s. Insoluble potassium perchlorate in each tube was precipitated by centrifugation at 20,000 × *g* for 5 min at 5 °C. The resulting supernatants were subjected to LC-ESI-MS/MS.

2.5. Procedures for method validation

To test specificity of the method, we prepared blank plasma spiked with the 11 standard molecules after the preparative procedures. Thus, blank plasma was prepared according Section 2.4, followed by the addition of 10 vol of the standard solution containing 100 ng/ml each to 90 vol of the prepared plasma to finally get blank plasma with the 11 molecules spiked at 10 ng/ml each. Similarly, in the calculation of LOD and limit of quantification (LOQ), blank plasma with the 11 standard molecules spiked at a concentration of 2 ng/ml each after the preparative procedures was used. Calibration lines were produced by injections of standard solutions in the range between 0.5 and 50 ng/ml (0.5, 2, 10, 20 and 50 ng/ml) for CQAs, FQAs, CA and FA, and 2 and 50 ng/ml (2, 10, 20 and 50 ng/ml) for dCQAs. In another experiment estimating quantitative recovery, the 11 molecules were spiked in the blank plasma to a final concentration of 20 ng/ml each, and the plasma was then subjected to the preparative procedures described in Section 2.4.

3. Results

3.1. Characteristics of product ions of CQAs, FQAs, dCQAs, CA and FA

Fang et al [5] succeeded in the molecular structural characterization of CQAs, FQAs, dCQAs, CA and FA in dried plums by LC-ESI-MS/MS (triple quadrupole) using acidic mobile phases and a negative ion mode, while Clifford et al [20] clarified the fragmentation patterns of CQAs, FQAs and dCQAs in a methanolic green coffee bean extract and a commercial cider by LC-ESI-MS^n (ion trap) using acidic mobile phases and a negative ion mode. Therefore, we determined the usefulness of an acidic carrier and negative ion ESI in this analysis. First, product ion spectra of three isomers each of standard CQAs, FQAs and dCQAs, and standard CA and FA were acquired in flow injection-MS/MS. Since, among the volatile acids tested, acetic acid provided the most abundant deprotonated molecular ions $[M-H]^-$ of all 11 molecules under negative ion ESI conditions, 50 mM aqueous acetic acid containing 20 vol% acetonitrile was used as a carrier.

When deprotonated molecular ions $[M-H]^-$ were used in scan measurements under a collision energy of 30 V, all three isomers of CQAs provided *m/z* 135, 173, 179 and 191 in the product ion spectra, corresponding to $[CA-CO_2-H]^-$, $[QA-H_2O-H]^-$, $[CA-H]^-$ and $[QA-H]^-$, respectively (data not shown). All three isomers of FQAs provided *m/z* 135, 173, 191 and 193, except for *m/z* 191 derived from 4-FQA (data not shown). The ions *m/z* 135 and 193 were assigned as $[FA-CO_2-CH_2-H]^-$ and $[FA-H]^-$, respectively. All three isomers of dCQAs provided *m/z* 135, 173, 179, 191 and 353 (data not shown). The ion *m/z* 353 was assigned as $[CQA-H]^-$. On the other hand, CA provided *m/z* 89, 107 and 135, while FA provided *m/z* 135, 149 and 179 (data not shown). The ions *m/z* 89, 107, 149 and 179 were assigned as $[CA-CO_2-CO-H_2O-H]^-$, $[CA-CO_2-CO-H]^-$, $[FA-CO_2-H]^-$ and $[FA-CH_2-H]^-$, respectively. These assignments were based on previous reports [6,20]. Among the characteristic product ions, three ions with abundant intensities in the product ion spectra were selected as diagnostic product ions, as they were *m/z* 135, 179 and 191 derived from the precursor *m/z* 353 for CQAs, 173, 191 and 193 derived from 367 for FQAs, 179, 193 and 353 derived from 515 for dCQAs, 89, 107 and 135 derived from 179 for CA, and 135, 149 and 179 derived from 193 for FA.

3.2. Effects of collision energy on intensities of diagnostic product ions

Since CQAs, FQAs, dCQAs, CA and FA may be present at extremely low concentrations in human plasma, it was considered that each of the diagnostic product ions must be detected with the highest sensitivity in ESI-MS/MS. Therefore, we examined the effects of a collision energy which greatly affects the generation of product ions on the intensities of each of the diagnostic product ions, using flow injection-ESI-MS/MS with negative ion mode and 50 mM aqueous acetic acid containing 20 vol% acetonitrile as a carrier.

Fig. 2 shows the relationships between collision energies and intensities of the diagnostic product ions derived from CQAs, FQAs and dCQAs. For the ion, *m/z* 353 → 135 of CQAs, a collision energy of 40 V provided the maximal intensities in all CQAs, despite the intensity being extremely small in 5-CQA (Fig. 2A). Therefore, the optimal collision energy for *m/z* 353 → 135 of CQAs was determined to be 40 V. The maximal intensities of the ion, *m/z* 353 → 179 of CQAs, were provided

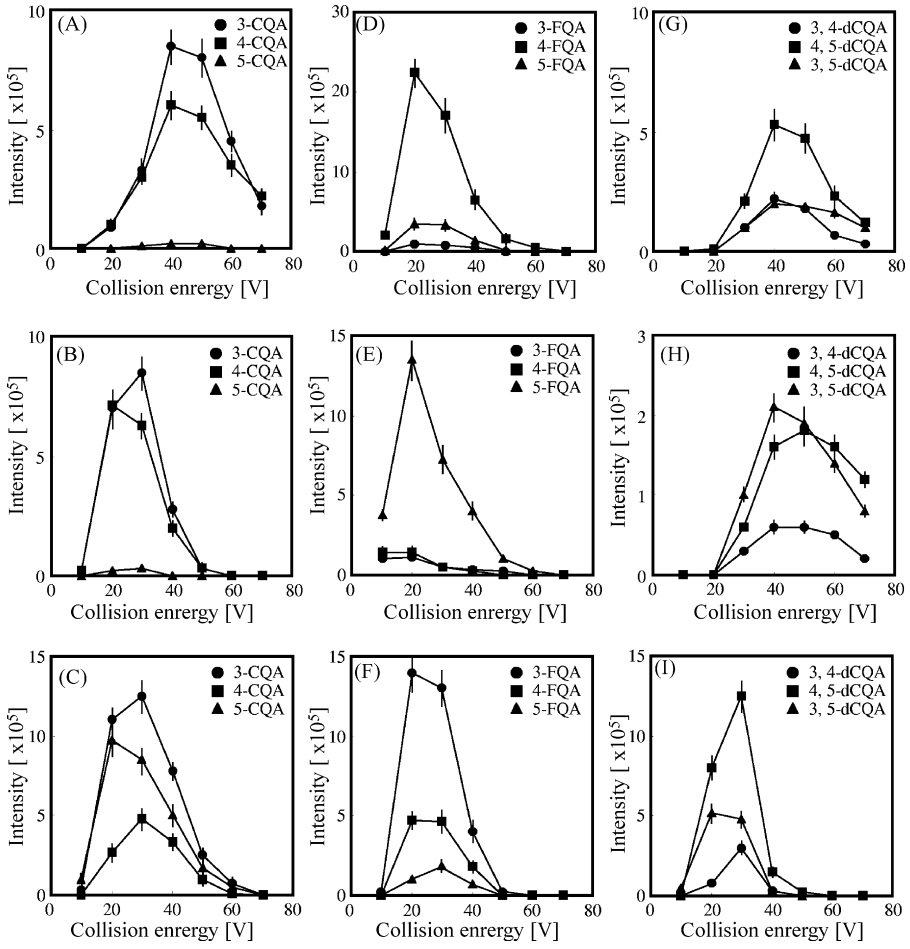

Fig. 2. Relationships between collision energies and intensities of diagnostic product ions of CQAs, FQAs and dCQAs in flow injection-MS/MS analysis with negative ion ESI. (A) *m/z* 353 → 135 of CQAs; (B) *m/z* 353 → 179 of CQAs; (C) *m/z* 353 → 191 of CQAs; (D) *m/z* 367 → 173 of FQAs; (E) *m/z* 367 → 191 of FQAs; (F) *m/z* 367 → 193 of FQAs; (G) *m/z* 515 → 179 of dCQAs; (H) *m/z* 515 → 191 of dCQAs; (I) *m/z* 515 → 353 of dCQAs. Error bar: SE ($n = 5$). Concentration: each 1 μg/ml. Experimental conditions: injection volume, 5 μl; carrier, 50 mM aqueous acetic acid containing 20 vol% acetonitrile at a flow rate of 0.2 ml/min; ion spray voltage, −4500 V; ion source heater temperature, 600 °C; ion source gas (N_2) for nebulizing, 50 psi; ion source gas (N_2) for drying solvent, 80 psi; curtain gas (N_2), 20 psi; declustering potential, −150 V; collision gas (N_2), 3×10^{-5} torr; collision energy, 10–70 V; collision exit energy, −10 V; scan range of Q3; *m/z* 50–400; scan rate, 200 amu/s.

at 30 V in 3-CQA and 5-CQA while a collision energy of 20 V provided the maximum in 4-CQA (Fig. 2B). Since the intensity at 30 V in 4-CQA was only slightly lower than that at 20 V, the optimal collision energy for *m/z* 353 → 179 of CQAs was determined to be 30 V. Similarly, the ion, *m/z* 353 → 191 of CQAs as depicted in Fig. 2C, was determined to be detected under a collision energy of 30 V. In FQAs, the diagnostic product ions, *m/z* 367 → 173, 367 → 191 and 367 → 193, yielded the strongest intensities in 4-FQA, 5-FQA and 3-FQA, respectively (Fig. 2D and F). The optimal collision energies for *m/z* 367 → 173, 367 → 191 and 367 → 193 of FQAs were determined to be 20, 20 and 30 V, respectively. All dCQAs, especially 3,4-dCQA, yielded relatively less abundance in the ion *m/z* 515 → 191, while the ion *m/z* 515 → 353 was provided with highly abundant intensities for all dCQAs (Fig. 2G–I). Collision energies for *m/z* 515 → 179, 515 → 191 and 515 → 353 of dCQAs were optimized into 40, 50 and 30 V, respectively. Fig. 3 shows the relationships between collision energies and intensities of the diagnostic product ions of CA and FA. The ions *m/z* 179 → 89, 179 → 107 and 179 → 135 of CA were determined to be detected under collision energies of 40, 30 and 20 V, respectively, although the intensities of *m/z* 179 → 89 and 179 → 107 were extremely low in abundance in the range of collision energies tested, compared with those of *m/z* 179 → 135 (Fig. 3A). For all diagnostic product ions of FA, collision energy of 20 V was selected, because they showed maximal intensities at 20 V (Fig. 3B). Also, *m/z* 353 → 191 for all CQAs, 367 → 193 for 3-FQA, 367 → 191 for 5-FQA, 367 → 173 for 4-FQA, 515 → 353 for dCQAs, 179 → 135 for CA, and 193 → 135 for FA were selected as target product ions in simultaneous MRM measurements for the quantitative analysis, because each of them exhibited the almost maximal intensities among the three diagnostic product ions under the given conditions.

3.3. Optimization of LC conditions

To elevate the sensitivity and qualitative performance of CQAs, FQAs, dCQAs, CA and FA in human plasma, the optimized ESI-MS/MS was connected to LC. Therefore, the LC separation of the 11 molecules was examined using a

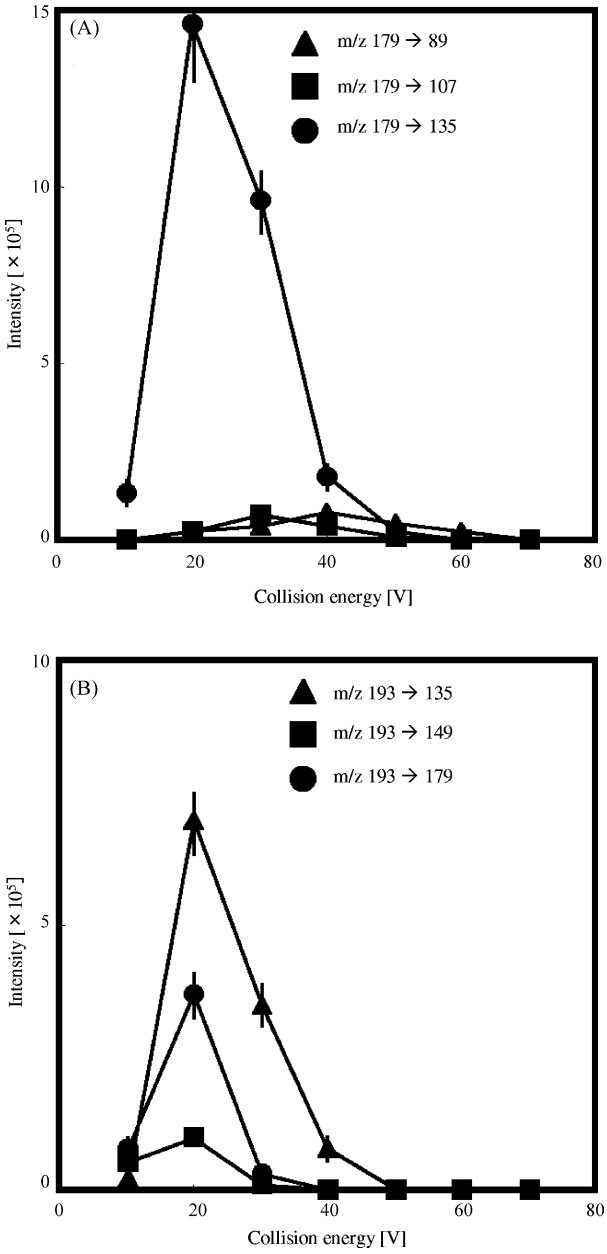

Fig. 3. Relationships between collision energies and intensities of diagnostic product ions of CA and FA in flow injection-MS/MS analysis with negative ion ESI. (A) CA; (B) FA. Error bar: SE ($n = 5$). Concentration: each 1 μg/ml. Experimental conditions as described for Fig. 2.

reversed-phase column. Solutions of 50 mM acetic acid with 3% acetonitrile and 50 mM acetic acid with acetonitrile were selected as mobile phases and were used under gradient elution conditions. Based on experiments using different gradient programs, we eventually found an optimal LC gradient program (Table 1) that allowed the mutual separation of the eleven molecules, including three isomers each of CQAs, FQAs and dCQAs, within ca. 32 min (the total run time was 60 min). A representative total ion chromatogram of the 11 standards (100 ng/ml each) under the optimized conditions is shown in Fig. 4, which was obtained using the target product ions for the quantitative analysis in the simultaneous MRM measurement.

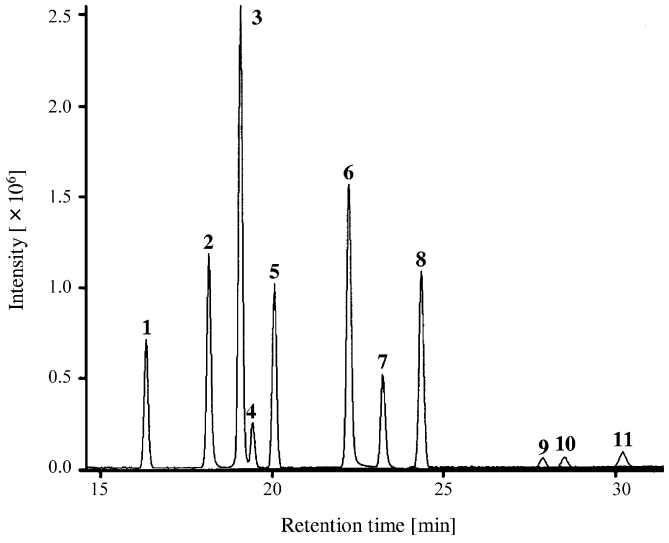

Fig. 4. LC-ESI-MS/MS MRM total ion chromatogram of standard CQAs, FQAs, dCQAs, CA and FA (100 ng/ml each) under optimized conditions. Peaks: correspond to numbers in Fig. 1. LC conditions: column, Inertsil ODS-2 (2.1 mm i.d. × 250 mm); column temperature, 35 °C; mobile phase: (A) 50 mM aqueous acetic acid containing 3 vol% acetonitrile; and (B) 50 mM acetic acid in acetonitrile; flow rate, 0.2 ml/min; gradient elution, see Section 2.2; injection, 5 μl. ESI-MS/MS conditions: collision energy and product ions; see Section 2.2; dwell time, 500 s each; other experimental conditions as described for Fig. 2.

As seen in Fig. 4, the isomers of CQAs, FQAs and dCQAs have different retention times and all 11 molecules elute in the order of 3-CQA, 5-CQA, CA, 4-CQA, 3-FQA, 5-FQA, 4-FQA, FA, 3,5-dCQA, 3,4-dCQA and 4,5-dCQA under these reversed-phase conditions. The %RSD for retention times obtained from six consecutive analyses of a 100 ng/ml solution for each of the 11 molecules were in the range of 0.04–0.16%. In addition to the selective detection of diagnostic product ions obtained by the simultaneous MRM measurement in ESI-MS/MS, the mutual separation of the 11 molecules by this LC separation enabled us to perform reliable qualitative analysis in unknown samples such as human plasma.

3.4. Method validation

We first confirmed that no peaks with the same retention times in the MRM chromatograms of the diagnostic product ions for the 11 standard molecules were observed in any of the plasma before the oral ingestion, which were collected from subjects #1 to #5, using the optimized LC-ESI-MS/MS method (data not shown). This implied that the plasma before the ingestion contained none of the 11 molecules and no endogenous molecules that interfered with the analysis. Next, blank plasma (collected from subject #1 before the ingestion) with the 11 molecules spiked at a concentration of 10 ng/ml each after the preparative procedures was analyzed to confirm that this method could detect all 11 molecules even when they co-existed with matrix components of the plasma. Those results provided evidence that each of the 11 molecules was detected in all MRM chromatograms, except for a MRM chromatogram of m/z 353 → 191 derived from 4-FQA (peak No. 7) which yielded almost no signal to

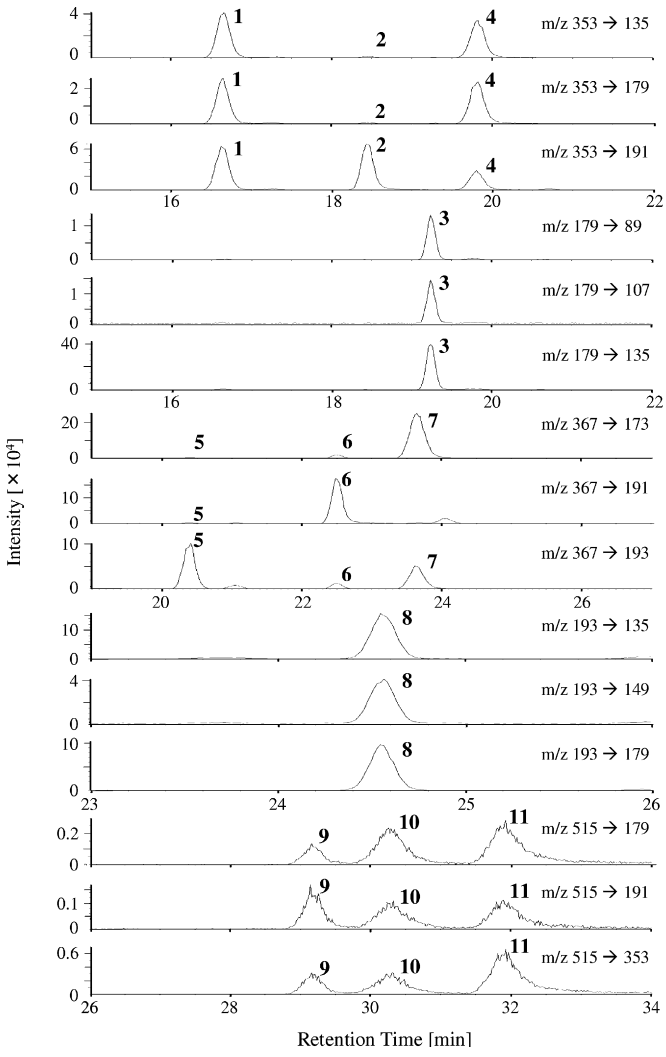

Fig. 5. LC-ESI-MS/MS MRM chromatograms of standard CQAs, FQAs, dCQAs, CA and FA spiked into blank plasma (10 ng/ml each). Blank plasma was collected from subject #1 before oral ingestion of a green coffee bean extract containing 300 mg CGAs. Peaks: correspond to numbers in Fig. 1. LC-ESI-MS/MS conditions: as described for Fig. 4. Diagnostic product ions: see Section 2.2.

noise abundance of the ion (Fig. 5). This demonstrates that the qualitative method used has specificity in the analysis of the 11 molecules in human plasma.

Using the product ions for the quantitative analysis (Section 2.2), LOD (S/N = 3), LOQ (S/N = 10), calibration lines and recoveries were examined. The analysis of the 11 molecules spiked in the blank plasma showed that the LOD and LOQ in the plasma ranged from 0.09 to 0.28 ng/ml and 0.30 to 0.92 ng/ml, respectively, for CQAs, FQAs, CA and FA, while the LOD and LOQ in plasma ranged from 0.66 to 0.88 ng/ml and 2.18 to 2.90 ng/ml, respectively, for dCQAs (Table 2). Based on these LOQ values, calibration lines were produced using standard solutions of 0.5–50 ng/ml for CQAs, FQAs, CA and FA, and of 2 and 50 ng/ml for dCQAs. All the determination coefficients were greater than 0.9989 (Table 2). Recoveries were obtained by analyzing the 11 standard molecules spiked into the blank plasma (collected from subject #1 before the ingestion) before the preparative procedure. In this recovery experiment, both matrix effects (ion suppression effect) and extraction efficiency were totally evaluated as overall recoveries. The average recoveries were in the range between 76.2 and 96.6% with the reproducibility (measurement errors ranged from ±1.6 to ±5.2%). Some recovery values were relatively low. However, we confirmed that they were reproducible and more than 75% of the spiked molecules were recovered. Taken together, we concluded that the sensitivity, linearity and recovery of this method are suitable for the rough estimation of quantitative values of the 11 molecules in the plasma.

3.5. Analysis of CQAs, FQAs, dCQAs, CA and FA in human plasma

The established method was applied to analyze the 11 molecules in human plasma obtained from subjects #1 to #5

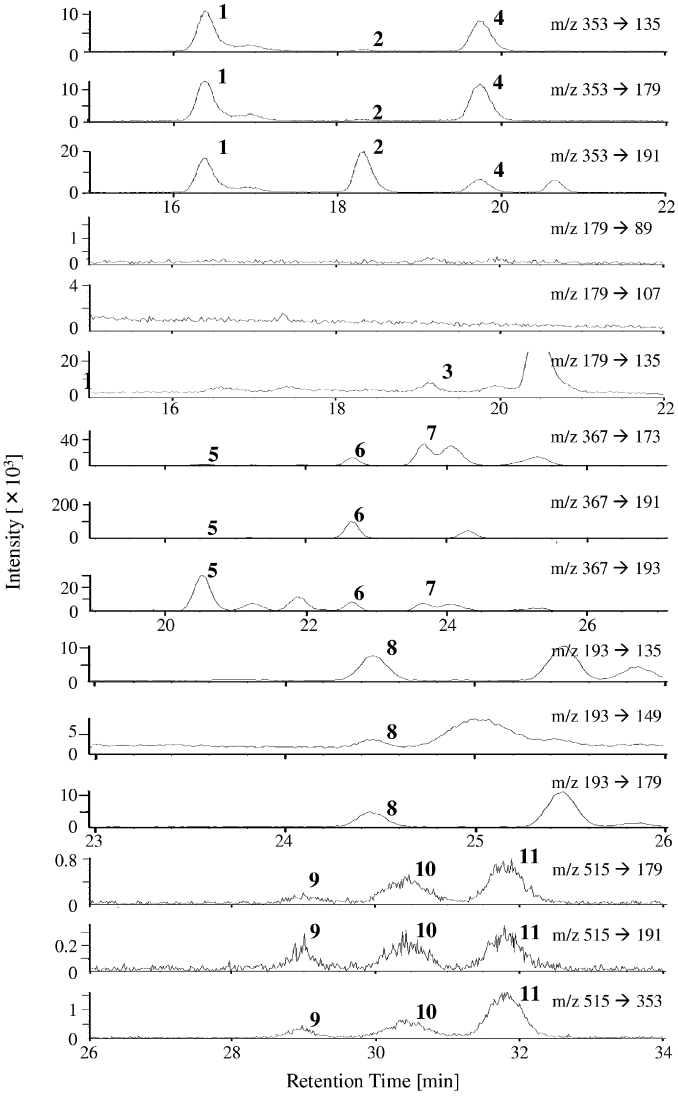

Fig. 6. LC-ESI-MS/MS MRM chromatograms of CQAs, FQAs, dCQAs, CA and FA in plasma 1 h after oral ingestion of 250 ml of a green coffee bean extract (300 mg CGAs). The plasma was collected from subject #1. Peaks: correspond to numbers in Fig. 1. LC-ESI-MS/MS conditions: as described for Fig. 4. Diagnostic product ions: see Section 2.2.

Table 2
LOD (S/N = 3), LOQ (S/N = 10), calibration lines and recoveries for 11 standard molecules under conditions of simultaneous MRM measurements in quantitative analysis

No.[a]	Molecule	LOD[b] (ng/ml)	LOQ[b] (ng/ml)	Calibration line[c]		Recovery[d] (%)
				Equation	R^2	
1	3-CQA	0.12	0.44	$y = 27850x - 3562$	0.9992	78.8 ± 5.2
2	5-CQA	0.09	0.31	$y = 54383x - 2938$	0.9992	89.4 ± 3.1
3	CA	0.09	0.30	$y = 144833x - 15867$	0.9994	83.6 ± 1.6
4	4-CQA	0.28	0.92	$y = 9315x + 304$	0.9996	91.4 ± 2.8
5	3-FQA	0.16	0.50	$y = 44967x - 3755$	0.9996	96.6 ± 1.7
6	5-FQA	0.16	0.53	$y = 72667x - 11198$	0.9996	85.9 ± 3.8
7	4-FQA	0.10	0.32	$y = 91017x - 6193$	0.9996	95.5 ± 3.2
8	FA	0.14	0.48	$y = 103667x - 7707$	0.9998	87.0 ± 2.8
9	3,5-dCQA	0.55	1.82	$y = 7485x + 857$	0.9993	76.2 ± 4.0
10	3,4-dCQA	0.58	1.91	$y = 6907x + 941$	0.9992	87.6 ± 3.0
11	4,5-dCQA	0.36	1.12	$y = 15633x + 2553$	0.9989	79.8 ± 2.2

[a] Corresponds to numbers in Fig. 1.
[b] Presented as concentrations in human plasma.
[c] R^2 is the determination coefficient, x is the injected amount of the standard molecule in ng/ml, and y is the peak area. Calibration lines were produced by injections of 0.5, 2, 10, 20 and 50 ng/ml for CQAs, FQAs, CA and FA and 2, 10, 20 and 50 ng/ml for dCQAs in standard solutions.
[d] Presented as mean ± SD. Six replicates of the blank plasma (subject #1) with the 11 standard molecules (20 ng/ml each) spiked before the preparative procedures were analyzed.

1 and 3 h after oral ingestion of 250 ml of a drink containing a green coffee bean extract (300 mg CGAs). As an example, the LC-ESI-MS/MS MRM chromatograms in the plasma of the subject #1 1 h after the ingestion are depicted in Fig. 6. In the case of subject #1 after 1 h plasma, distinct peaks with the same retention times as those in the blank plasma with the spiked standards were detected for most of the diagnostic product ions in the corresponding MRM chromatograms (Figs. 5 and 6). Further, relative intensities of the diagnostic product ions derived from the 11 molecules in subject #1 after 1 h plasma were almost in agreement with those in the blank plasma with the spiked standards (Table 3). Peaks corresponding to m/z 179 → 89 and 179 → 107 of CA (peak No. 3) were not detected (Figs. 5 and 6), probably due to a much lower concentration of CA in the plasma than the spiked concentration (10 ng/ml) in the blank plasma. Also, all diagnostic product ions originated from dCQAs had less abundant intensities (Figs. 5 and 6). Similar results were observed in the other plasma samples. This demonstrates the existence of all 11 molecules (three isomers each of CQAs, FQAs, dCQAs, and their metabolites CA and FA) in the plasma after ingestion of the coffee bean extract containing CGAs. In addition to the 11 known molecules, co-existing peaks, such as observed in MRM chromatograms of m/z 179 → 135 of CA, m/z 367 → 173, 367 → 191 and 367 → 193 of FQAs and m/z 193 → 135, 193 → 149 and 193 → 179 of FA, other than the diagnostic ion peaks (peaks No. 3 and 5–8) seemed to result from metabolites of CGAs ingested since those peaks were not detected in the blank plasma with the spiked standards (Figs. 5 and 6). Especially, the two peaks that have slightly longer retention times (ca. 24.1 and 25.3 min) than the diagnostic ions of 4-FQA in m/z 367 → 137 and 367 → 193 MRM chromatograms may be derived from metabolites which are close in structure to 4-FQA having the structures of both FA and QA moieties within the molecule.

Table 3
Relative intensities of diagnostic product ions derived from CQAs, FQAs, dCQAs, CA and FA under optimized LC-ESI-MS/MS MRM conditions

No.[a]	Molecule	Relative intensity in % (m/z value)	
		Blank plasma with spiked standards[b]	Plasma after oral ingestion[c]
1	3-CQA	100 (191) 80 (179) 66 (135)	100 (191) 78 (179) 62 (135)
2	5-CQA	100 (191) 2 (135) 2 (179)	100 (191) 1 (135) 1 (179)
3	CA	100 (135) 3 (89) 3 (107)	100 (135) 0 (89) 0 (107)
4	4-CQA	100 (179) 71 (135) 54 (191)	100 (179) 68 (135) 53 (191)
5	3-FQA	100 (193) 5 (173) 3 (191)	100 (193) 7 (173) 2 (191)
6	5-FQA	100 (191) 12 (173) 7 (193)	100 (191) 12 (173) 8 (193)
7	4-FQA	100 (173) 19 (193) 0 (191)	100 (173) 29 (193) 0 (191)
8	FA	100 (135) 61 (179) 26 (149)	100 (135) 61 (179) 21 (149)
9	3,5-dCQA	100 (353) 48 (191) 41 (179)	100 (353) 40 (191) 20 (179)
10	3,4-dCQA	100 (353) 76 (179) 33 (191)	100 (353) 80 (179) 40 (191)
11	4,5-dCQA	100 (353) 39 (179) 16 (191)	100 (353) 50 (179) 20 (191)

[a] Corresponds to numbers in Fig. 1.
[b] Standard CQAs, FQAs, dCQAs, CA and FA (10 ng/ml each) were spiked into blank human plasma.
[c] Human plasma was collected from subject #1 1 h after oral ingestion of 250 ml of a drink containing a green coffee bean extract (300 mg CGAs).

Table 4
Concentrations of CQAs, FQAs, and FA in human plasma 1 and 3 h after oral ingestion of a drink containing a green coffee bean extract (300 mg CGAs)

No.[a]	Molecule	Concentration in plasma (ng/ml)[b]	
		1 h after ingestion	3 h after ingestion
1	3-CQA	3.91 ± 1.22	2.50 ± 2.13
2	5-CQA	7.39 ± 2.64	4.05 ± 3.30
4	4-CQA	6.81 ± 2.31	4.08 ± 3.46
5	3-FQA	6.64 ± 1.33	3.30 ± 1.72
6	5-FQA	13.87 ± 3.44	7.01 ± 3.64
7	4-FQA	3.80 ± 1.56	2.78 ± 1.39
8	FA	0.89 ± 0.44[c]	N.Q.[d]

[a] Corresponds to numbers in Fig. 1.
[b] Presented as mean ± SD for five plasma collected from five different subjects.
[c] Regarded as a concentration being 0.00 in case that a calculated value was less than LOQ.
[d] Not quantified (less than LOQ).

The quantitative analysis of the 11 molecules followed the qualitative analysis described above. For CQAs and FQAs, we could get quantitative values that were within the range of calibration lines in all plasma tested. As for CA, some of the plasma tested showed more than its LOD but less than LOQ values while the others were less than LOD (=not detected). FA was detected over its LOQ value in most of the plasma 1 h after the ingestion while it was less than its LOQ value in all plasma after 3 h. In most of the plasma tested, dCQAs were detected but almost equivalent to their LOQ values. Based on these facts, Table 4 shows average concentrations of CQAs, FQAs and FA in the plasma after 1 and 3 h, although only an average concentration of FA in the plasma after 3 h is not shown. In the plasma after 1 h, the average concentration of 5-FQA was the highest, followed by the order of 5-CQA, 4-CQA, 3-FQA, 3-CQA, 4-FQA and FA. Although their concentrations in the plasma after 3 h were slightly decreased, the order was almost the same as that in the plasma after 1 h. As results of the quantitative analysis, the following phenomena were found: (1) the concentrations of CGAs in the plasma were remarkably lower than those in the drink ingested. (2) Small but distinct FA levels were detected in the plasma at a relatively early stage after the ingestion of CGAs. (3) The concentrations of FQAs in the drink were relatively low while those in the plasma were relatively high, especially for 5-FQA, compared with those of CQAs.

4. Discussion

In this study, we optimized LC-ESI-MS/MS conditions for the simultaneous analysis of a total of 11 molecules, three isomers each of CQAs, FQAs and dCQAs, and their metabolites, CA and FA, in human plasma. We used that technique to characterize plasma collected from healthy human subjects 1 and 3 h after oral ingestion of an extract of green coffee beans containing CGAs. The LC separation is chromatographically achieved by combining a reversed-phase Inertsil ODS-2 column with a gradient elution system using two mobile phases. Under the optimized LC conditions, three isomers each of CQAs, FQAs and dCQAs are mutually separated from each other as well as from CA and FA. The ESI-MS/MS detection with a negative ion mode employs simultaneous MRM measurements of three diagnostic product ions derived from each precursor ion $[M-H]^-$ of the 11 molecules, based on examining the effects of collision energies on the intensity of each of those diagnostic product ions. Under the optimized conditions of collision energies, the 11 standards (100 ng/ml each) spiked into blank human plasma yielded characteristic peaks in corresponding MRM chromatograms. On the other hand, when a product ion for each of the 11 molecules is selected in terms of the peak intensities under the optimized conditions, it seems that the sensitivity, linearity and recovery are acceptable to use this method for the quantitative analysis of the 11 molecules in the plasma. Among the 11 molecules, 3-CQA, 3,5-dCQA and 4,5-dCQA had relatively lower recovery percentages. Since the recovery test in this study has been conducted as overall recovery, we do not currently understand which of the matrix effect (ion suppression effect) or extraction efficiency causes such relatively low recoveries. Also we do not currently use an internal standard in the method. If we knew the cause(s) of lower recoveries and/or adopted the internal standard procedure, more satisfactory recovery percentages might be acquired. However, it should be noted that the present validation data show the availability of the current method for the rough estimation of concentrations in the plasma. Therefore, this method can be used for the simultaneous qualitative and quantitative analysis of CQAs, FQAs, dCQAs, CA and FA in plasma.

The LC-ESI-MS/MS method provides information about reversed-phase retention times and three diagnostic product ions (presence or absence and relative intensities if present) for each of the 11 molecules, which enables their reliable qualitative analysis of the extremely low levels of the 11 molecules in human plasma. In addition, this method is also useful for the quantitative analysis when the abundant product ion among the three diagnostic ions for each of the 11 molecules is selected in the simultaneous MRM measurement. The method developed in this study is superior to the previously reported LC-ECD [11,12] and LC-UVD [13,15,16] techniques which provide only reversed-phase retention times. If higher levels of the 11 molecules were present in human plasma, we could have directly used product ion scan measurements in LC-ESI-MS/MS to analyze them, as did previous studies [6,20]. Another previously reported method used GC–MS scan measurements [14] for the analysis but was too insensitive. LC-ESI-MS selected ion monitoring [10] and LC-ESI-MS/MS MRM using only one product ion [17,18] have high sensitivity but provide little reliable information on the qualification. Therefore, the superiority of our method is evident compared with the conventional ones. However, we also think the method for the quantitative analysis must be further improved. That is because the present method can be practically available for determining concentrations of CQAs and FQAs in the plasma but is not able to determine those of CA and dCQAs. The method shows the highest sensitivity in CA of all 11 molecules, but indeed it was difficult to quantify the level of CA in the plasma after the ingestion. As for FA, this method is useful for the determination in the plasma at a relatively early stage, when the concentration of FA is high, but is not useful

at a relatively late stage. We need to increase the sensitivity of detection of FA to understand the pharmacokinetics of FA in the plasma after the ingestion. Thus, further increasing the detection sensitivity for CA, dCQA and FA is our future goal.

Our application reveals the existence of all 11 molecules (three isomers each of CQAs, FQAs, dCQAs, and their metabolites CA and FA) in the plasma after ingestion of the coffee bean extract containing CGAs, due to the superiority of this method from the viewpoints of its high sensitivity and qualitative performance. Among these results, it should be emphasized that CGAs, FQAs and dCQAs have been successfully detected in human plasma for the first time. Thus, low but distinct levels of CQAs were detected in human urine after oral ingestion of CQAs [10,13,14], and 5-CQA was detected in plasma from other animals after oral ingestion of large amounts of 5-CQA. This suggested the absorption of CQAs into human plasma after the ingestion, but there was no direct evidence regarding this. There were no previous reports detecting FQAs and/or dCQAs not only in human plasma but also in human urine or other animal plasma/urine. Therefore, our results indicate that at least FQAs and dCQAs are directly absorbed into human blood after the ingestion. As for FQAs, we think that most of those detected in the plasma may be absorbed as intact molecules. However, it is possible that some of the FQAs detected in the plasma may be metabolized via methylation of CQAs directly absorbed into the plasma, because CA seems to be converted to FA after absorption [9]. Similarly, the reason for the relatively high concentrations of FQAs in plasma despite the low concentrations in the drink may be ascribed to the fact that FQAs are readily produced in the plasma or other tissues after the adsorption of CQAs into the plasma. This aspect should be clarified in the near future because it might be closely related to biological and pharmacological effects of coffee drinks, such as improvement of human hypertension [3,4]. Other results regarding CA and FA are consistent with previous reports describing the existence of CA and FA in human plasma [10–12]. Since glucuronate and/or sulfate conjugates of CA and FA were previously detected [10,11], it is possible that other metabolites, such as glucuronide and/or sulfate conjugates of CQAs, FQAs and dCQAs, may occur in human plasma, as in the case of green tea catechins [21]. At least, we have found candidate metabolites which seem to be close in structure to 4-FQA. This method, when combined with enzymatic treatment with β-glucuronidase and sulfatase to also analyze conjugated forms, is a powerful tool to understand biological and pharmacological effects of CGAs, such as improvement of human hypertension disorders.

In conclusion, we have established a new method to simultaneously analyze 11 CGA-related molecules in human plasma after the ingestion of an extract of green coffee beans which contain CGAs. The method uses reversed-phase LC combined with simultaneous ESI-MS/MS MRM measurements of product ions derived from each of the 11 molecules. The LC-ESI-MS/MS analysis uses optimized collision energies to detect each product ion and provides a sensitive and qualitative/quantitative analysis of all 11 molecules. The method was shown to be useful for analysis of those 11 molecules in human plasma after oral ingestion of a coffee bean extract. Using this method, we detected three isomers each of CQAs, FQAs and dCQAs together with CA and FA in human plasma for the first time. This novel method will be useful to understand the diverse biological and pharmacological effects of CGAs, such as their effects on human hypertension, and to study health care foods to prevent so-called lifestyle-related diseases.

Acknowledgements

We would like to express our cordial gratitude to Dr. Katsumi Kita and Dr. Ichiro Tokimitsu of the Kao Corporation for their invaluable discussion and encouragement of this study. Our sincere thanks are also due to Ms. Namii Shimizu of the Kao Corporation for her technical help.

References

[1] M.T. Huang, R.C. Smart, C.Q. Wong, A.H. Conney, Cancer Res. 48 (1988) 5941.
[2] Y. Kono, K. Kobayashi, S. Tagawa, K. Adachi, A. Ueda, Y. Sawa, H. Shibata, Biochim. Biophys. Acta 1335 (1997) 335.
[3] I. Saito, T. Tsuchida, T. Watanabe, Y. Arai, Y. Mitsui, W. Okawa, Y. Kajihara, Jpn. J. Med. Pharm. Sci. 47 (2002) 67.
[4] R. Ochiai, H. Jokura, A. Suzuki, I. Tokimitsu, M. Ohishi, N. Komai, H. Rakugi, T. Ogihara, Hypertens. Res. 27 (2004) 731.
[5] N. Fang, S. Yu, R.L. Prior, J. Agric. Food Chem. 50 (2002) 3579.
[6] B.D. Whitaker, J.R. Stommel, J. Agric. Food Chem. 51 (2003) 3448.
[7] M.N. Clifford, S. Knight, B. Surucu, N. Kuhnert, J. Agric. Food Chem. 54 (2006) 1957.
[8] A. Tolonen, T. Joutsamo, S. Mattlla, T. Kamarainen, J. Jalonen, Phytochem. Anal. 13 (2002) 316.
[9] K. Azuma, K. Ippoushi, M. Nakayama, H. Ito, H. Higashio, J. Terao, J. Agric. Food Chem. 48 (2000) 5496.
[10] P. Cremin, S.K-. Karakas, A.L. Waterhouse, J. Agric Food Chem. 49 (2001) 1747.
[11] M. Nardini, E. Cirillo, F. Natella, C. Scaccini, J. Agric. Food Chem. 50 (2002) 5735.
[12] S.M. Wittemer, M. Ploch, T. Windeck, S.C. Muller, B. Drewelow, H. Derendorf, Phytomedicine 12 (2005) 28.
[13] M.R. Olthof, P.C. Hollman, M.B. Katan, J. Nutr. 131 (2001) 66.
[14] M.R. Olthof, P.C. Hollman, M.N. Buijsman, J.M. van Amelsvoort, M.B. Katan, J. Nutr. 133 (2003) 1806.
[15] M. Takenaka, T. Nagata, M. Yoshida, Biosci. Biotech. Biochem. 64 (2000) 2689.
[16] H. Yang, B. Yuan, L. Li, H. Chen, F. Li, J. Chromatogr. Sci. 42 (2004) 173.
[17] S. Lafay, A. Gil-Izquierdo, C. Manach, C. Morand, C. Besson, A. Scalbert, J. Nutr. 136 (2006) 1192.
[18] M.P. Gonthier, M.A. Verny, C. Besson, C. Remesy, A. Scalbert, J. Nutr. 133 (2003) 1853.
[19] Y. Masukawa, Y. Matsui, N. Shimizu, N. Kondou, H. Endou, M. Kuzukawa, T. Hase, J. Chromatogr. B 834 (2006) 26.
[20] M.N. Clifford, K.L. Johnston, S. Knight, N. Kuhnert, J. Agric. Food Chem. 51 (2003) 2900.
[21] M.K. Piskula, J. Terao, J. Nutr. 128 (1998) 1172.