Attomole Catechins Determination by Capillary Liquid Chromatography with Electrochemical Detection

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Attomole quantities of catechins were determined by a capillary liquid chromatography system with electrochemical detection (CLC-ECD) and the system is applied to the determination of catechins in human plasma. The eight catechins: catechin (C), epicatechin (EC), gallic catechin (GC), epigallocatechin (EGC), catechin gallate (Cg), epicatechin gallate (ECg), gallic catechin gallate (GCg), and epigallocatechin gallate (EGCg), were separated within 10 min using a capillary column (0.2 mm i.d.) and a mobile phase of phosphoric acid (85%)-methanol-water (0.5:27.5:72.5, v/v/v), and were detected at +0.85 V vs. Ag/AgCl. Peak heights were found to be linearly related to the amount of catechins injected, from 200 amol to 500 fmol ($r > 0.998$). The detection limits of the catechins were 61 amol for EGC, 75 amol for EC, 54 amol for GC, 61 amol for C, 67 amol for GCg, 75 amol for EGCg, 75 amol for ECg and 89 amol for Cg ($S/N = 3$). Because the present method is highly sensitive and allows facile pretreatment for plasma sample, the time courses of concentrations of catechins (GCg, EC, EGCg, ECg, and Cg) and their conjugates in human plasma obtained from a 10 μ l plasma sample after ingestion of green tea could be determined.

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Introduction

Catechins (catechin (C), epicatechin (EC), gallic catechin (GC), epigallocatechin (EGC), catechin gallate (Cg), epicatechin gallate (ECg), gallic catechin gallate (GCg), and epigallocatechin gallate (EGCg)) in teas and foods are of interest for potential applications in cancer and coronary heart disease prevention. These compounds have been found to exert antioxidative,¹ antitumoral,² and antimutagenic effects.³ To elucidate the actual kinetic metabolism of catechins in the human body, we must develop a highly sensitive method that enables separation and determination.

Analytical methods that are suitable for the measurement of catechins from biological samples have mainly been based on high-performance liquid chromatography (HPLC) with ultraviolet detection (HPLC-UV).^{4,5} However, HPLC-UV lacks both the selectivity and sensitivity required when analyzing low levels of catechins in biological samples. Analytical methods with lower detection limits, based on HPLC with fluorometric (FL),⁶ chemiluminescence (CL),⁷ electrochemical (ECD),⁸⁻¹⁰ and mass spectrometric (MS) detection,¹¹ have been introduced. LC with MS (LC-MS) appeared to be a sensitive method for determining catechins; however, LC-MS requires expensive equipment and it has proven difficult to establish whether LC-MS shows enough precision. When the determination of catechins was carried out by HPLC with ECD (HPLC-ECD) using a pulseless pump and a wall-jet type electrochemical cell, the data with high precision were obtained.¹²⁻¹⁴

Moreover, to discuss the detail metabolic pathway containing

enterohepatic circulation, absorption by the gastrointestinal tract, and drug interactions, one must develop a determination method which uses very small amounts of plasma samples. However, because of the lower sensitivity of those analytical methods,^{6,7,9,10} a 100 to 500 μ l plasma sample was required for determining catechins in plasma. For various kinetic studies, it is desired to develop a more sensitive method for determining catechins in plasma with very small sample size from the viewpoint of bioethics to reduce the burdens for human subjects and experimental animals.

Microcolumn liquid chromatography, also known as capillary liquid chromatography (CLC), was developed at the same time as wide-bore (conventional) HPLC. However, slow progress in developing necessary instrumentation has kept CLC more of a research topic than a practical tool in the laboratory. Only recently, due to the availability of commercial capillary columns and progress in instrumentation, has it been possible to study of the ability of CLC as an alternative to conventional HPLC. CLC, with packed 0.1–0.5 mm inner diameter (i.d.) columns, has been established as an alternative technique to conventional-sized HPLC.¹⁵ The most critical point for increasing the sensitivity of ECD is the analyte concentration on the surface of the working electrode. If diluting the analyte concentration, which is caused by diffusion in the pathway, can be avoided, highly sensitive electrochemical detection is possible. Because such diffusion is more likely to occur in a conventional column or in a microbore column rather than in a capillary column, CLC is suitable for the development of a highly sensitive system.

In this study, we developed a CLC-ECD system for determining attomole quantities of catechins. By optimizing system composition, CLC is open to further improvement in performance capacity. In order to obtain optimum performance

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Fig. 1 Catechin structures. a. Molecular weights were calculated using Advanced Chemistry Development (ACD/Labs) Software V8.14 for Solaris.

in CLC, one should minimize peak band broadening outside the column, such as in pipes in the flow cell. Because peak volume is directly related to the square of the column diameter, the effect of the band broadening sources in an HPLC instrument is reduced with smaller columns and injection volumes. Therefore the present CLC-ECD would be given good reproducibility in analysis of biological sample. Thus, the CLC-ECD method developed for determining catechins was validated and applied to the determination of catechins in 10 μ l of human plasma. Five catechins and their conjugates in human plasma subsequent to the ingestion of one can of commercial green tea were quantified by the present methods.

Experimental

Materials and reagents

(-)-Gallocatechin (GC, >98%), (-)-epigallocatechin (EGC, >98%), (-)-catechin (C, >98%), (-)-epigallocatechin gallate (EGCg, >98%), (-)-epicatechin (EC, >98%), (-)-gallocatechin gallate (GCg, >98%), (-)-epicatechin gallate (ECg, >98%), and (-)-catechin gallate (Cg, >98%) were obtained from Kurita Industrial (Tokyo, Japan). These catechin structures are shown in Fig. 1. Other reagents and HPLC grade solvents were obtained from Wako Pure Chemical Industries (Osaka, Japan). Commercial canned green tea samples were purchased from Kirin Beverage (Tokyo, Japan). β -Glucuronidase and sulfatase were obtained from Sigma (St. Louis, MO, USA).

CLC-ECD system and conditions

The construction of CLC-ECD system is described in "Construction of CLC-ECD system" in Results and Discussion section. A deaerated phosphoric acid (85%)-methanol-water mixture (0.5:27.5:72.5, v/v/v) solution served as the mobile phase at a flow rate of 4.0 μ l min⁻¹. The detection potential for monitoring catechins was set at +0.85 V vs. Ag/AgCl.

Preparation of tea sample

In order to prepare a test solution, we mixed a sample of commercial canned green tea (10 ml) with an equivalent amount of phosphoric acid (85%)-methanol-water (0.5:27.5:72.5, v/v/v) mixture and sonicated the combination for 1 min, diluted 500 times again with the above mixture, and then filtered through a

Fig. 2 Capillary liquid chromatography with electrochemical detection (CLC-ECD) system (A) and schematic diagrams of electrochemical flow cell (B). (A) Column inlet (a) and outlet (b) were improved as in Figs. 3A and B, respectively. (B) (1) Glassy carbon electrode (ϕ 0.7 mm), (2) reference electrode, (3) counter electrode, (4) fused silica capillary tubing (0.05 mm i.d., 0.3 mm o.d.), (5) Teflon[®] tube, (6) nut and ferrule, (7) gasket.

0.45- μ m membrane filter. Fifty nanoliters of the test solution aliquots were injected into the CLC system.

Sample preparation of free and conjugated form catechins in plasma

A healthy adult subject (subject T, 30-years-old male, 98 kg body wt, smoker) was studied upon receipt of his informed consent. Before commencement of the trial, the protocol and the subject's informed consent were submitted to and approved by the Institutional Human Research Committee of Tokyo University of Pharmacy and Life Sciences. All plasma samples were obtained from subject T, who was not permitted the intake of tea or tea-related beverages for at least 1 week prior to the experiment. After fasting for 12 h, 340 ml commercial canned green tea was orally ingested and no other beverages were taken except water. To collect a 20- μ l blood, the finger was pricked with a lancing device (Hoechst Marion Roussel). The plasma obtained from 20 μ l of blood was used for plasma catechins determination. Plasma samples were taken before tea ingestion and 1, 1.5, 2, 3, 4, and 6 h after ingestion. Plasma samples were stored at -30°C until analysis.

Ten microliters of plasma were mixed with 10 μ l 0.4 M phosphate buffer (pH 3.6) containing 2% ascorbic acid and 0.1% ethylenediaminetetraacetic acid disodium salt, and 30 μ l 0.1 M phosphate buffer (pH 6.8) containing 500 U β -glucuronidase, 40 U sulfatase, and 5.0 pmol propyl gallate as the internal standard. The mixture was incubated at 37°C for 45 min to digest conjugated forms of catechins completely.^{8,10} Catechins in the enzyme-hydrolyzed solution were extracted three times with 50 μ l aliquots of ethyl acetate. The collected ethyl acetate was evaporated to dryness using a nitrogen stream. The dry residue was dissolved in 10 μ l phosphoric acid (85%)-methanol-water (0.5:27.5:72.5, v/v/v) solution to obtain a test solution for total amounts of free and conjugated form catechins. A test solution for free form catechins was prepared in a similar way without the enzyme digestion: ten microliters of plasma were mixed with the above-mentioned solution

Table 1 Effects of wetted material on CLC-ECD pump (material in the pathway) and thickness of gasket in flow cell on baseline noise level and detection limit of epicatechin

Pump	Thickness of gasket/ μm	Width of baseline noise/pA	Detection limit of EC/amol
MP 711 (stainless)	25	0.42	260
MP 710i (PEEK)	25	0.34	180
MP 710i (PEEK)	15	0.29	75

without enzyme, catechins in the mixture were extracted with ethyl acetate, and the collected ethyl acetate was evaporated to dryness using a nitrogen stream. Fifty nanoliters of test solution aliquots were injected into the CLC-ECD system.

Results and Discussion

Construction of CLC-ECD system

The CLC-ECD system (Fig. 2A) comprised of an MP711 pump (stainless is used in the pathway; GL Science, Tokyo, Japan) or an MP710i pump (polyetheretherketone (PEEK) is used in the pathway; GL Science), a C4-0004-05 microbore internal injector (Valco, Houston, TX, USA), an Inertsil ODS-3 Capillary EX-Nano column (150 mm $\times$ 0.2 mm i.d., 3 μm , GL Science), and a Decade electrochemical detector with column oven (Antec Leyden, Zoeterwoude, The Netherlands). A commercially available electrochemical cell ($\mu\text{VT-03}$, Antec Leyden) was constructed from a glassy carbon working electrode (ϕ 0.7 mm), an Ag/AgCl reference electrode, and a stainless steel auxiliary electrode (Fig. 2B). A 50 nl standard catechin solution was injected into the capillary ODS column maintained at 50°C.

One of the most important objectives is to reduce dead volume in the flow system of CLC system for highly sensitive detection. In order to make the dead volume smaller, while a connecting capillary fused silica tube is usually necessary between the injector and a conventional capillary column which has 1/16 inch female inlet configuration, the Inertsil ODS-3 Capillary EX-Nano column was directly connected with the injector in the present CLC system, as shown in Fig. 3A. By using a capillary tube of 0.3 mm outer diameter and 100 mm length, the column was connected with the electrochemical cell. Figure 3B shows the comparison of the outlet structure to the usual type. As shown in Fig. 3B, the capillary fused silica tube was directly connected to the electrochemical cell so that dead volume was almost eliminated.

In the HPLC-ECD, the electrolysis of impurities present in the mobile phase and in contaminants and corrosions from the pathway of the LC system enhance the chromatographic baseline noises. A comparison was made of the effects of the pathway including pump and tubes on chromatographic baseline noise and the detection limits of catechins using MP710i and MP711 pumps, that use PEEK and stainless in the pathway in the pump, respectively. As shown in Table 1, chromatographic baseline noise of CLC-ECD using MP710i pump is smaller than that using MP711 pump. The detection limits of EC, as an example, are shown in Table 1. These results indicate that MP-710i was a suitable pump for the CLC-ECD system.

The cell volume in electrochemical flow cell is an important factor for the highly sensitive determination by CLC-ECD. The current using a flow cell is expressed by Eq. (1):^{16,17}

A Column inlet

B Column outlet

Fig. 3 Schematic diagrams of the improvement on connection in column inlet (A) and outlet (B) of CLC-ECD system: (a) capillary column, (b) outlet port of injector to column (0.15 mm i.d.), (c) connection tube, (d) inlet port to ECD cell (0.3 mm i.d.), (e) fused silica capillary tube (0.05 mm i.d., 0.375 mm o.d.), (f) fused silica capillary tube (0.05 mm i.d., 0.3 mm o.d.), (g) flow cell, (h) union.

$$i = 1.47n_e F U^{1/3} (DA/h)^{2/3} C \quad (1)$$

where n_e is the number of electrons transferred in the redox reaction, F is Faraday's constant, U is the volume flow-rate, D is the analyte diffusion coefficient, A is the working electrode surface area, h is the height of the thin-layer channel (*i.e.*, the thickness of the gasket), and C is the analyte concentration. Therefore, when the thickness of the gasket was decreased, the cell current (i) was expected to increase. However, as suggested by Rocklin *et al.*, there is a limit to decreasing a gasket thickness depending on the flow cell design and the characteristics of the gasket. In this study, the effects of the thickness of a gasket in the flow cell on the chromatographic baseline noises level and on the detection limit of catechins were examined. As shown in Table 1, the detection limit with 15 μm -gasket showed a lower value than that with 25 μm -gasket.

Optimization of CLC-ECD conditions

Hydrodynamic voltammograms (Fig. 4) were measured in order to select the optimal detection potential for determining of catechins. Catechins were oxidized at potentials of more than +0.3 V vs. Ag/AgCl. Two oxidation waves, one at +0.5 – 0.6 V vs. Ag/AgCl and the other at +0.85 V vs. Ag/AgCl, were observed in the hydrodynamic voltammogram. For potentials more positive than +0.9 V vs. Ag/AgCl, sensitivity was increased, but reproducibility was decreased, possibly due to contamination of the electrode surface by oxidation products. Therefore, for highly sensitive determination without loss of selectivity and reproducibility, the value +0.85 V vs. Ag/AgCl was adopted for the present study.

An examination was made of how the ratio of water to methanol in the mobile phase influenced the separation for the determinations of catechins. The larger the content of water, the greater was the separation of these peaks. To determine catechins in human plasma with adequate resolution, within a short time, a mixture of methanol-water (27.5:72.5) was chosen for the most suitable mobile phase, and the column temperature,

Table 2 Intra-day accuracy, precision, and calibration curve linearity for the measurement of catechins peaks

Catechin	Nominal amount/ fmol	Determined amount/ fmol	Accuracy RE, %	RSD, % ($n = 5$)	Linearity			
					Regression equation ^{a,b}	r^b	fmol	Range ^b / ng ml ⁻¹ – μ g ml ⁻¹
GC	25.0	26.1	4.4	2.2	$y = 6.59x + 39.6$	0.998	0.2 – 500	1.23 – 3.06
EGC	25.0	25.0	0.1	1.5	$y = 7.26x + 42.1$	0.998	0.2 – 500	1.23 – 3.06
C	25.0	24.3	-2.8	1.8	$y = 6.10x + 33.5$	0.999	0.2 – 500	1.16 – 2.91
EGCg	25.0	24.5	-2.0	1.6	$y = 6.34x + 37.2$	0.998	0.2 – 500	1.83 – 4.58
EC	25.0	25.7	2.8	1.9	$y = 5.84x + 31.7$	0.999	0.2 – 500	1.16 – 2.91
GCg	25.0	25.5	2.2	1.9	$y = 7.57x + 49.7$	0.998	0.2 – 500	1.83 – 4.58
ECg	25.0	25.4	1.4	1.8	$y = 5.23x + 35.2$	0.998	0.2 – 500	1.77 – 4.42
Cg	25.0	26.1	4.4	1.8	$y = 3.80x + 24.2$	0.998	0.2 – 500	1.77 – 4.42

a. x and y indicate catechin amount (fmol) and peak current height (pA), respectively. b. The number of plots used to show linearity in the concentration range was 14.

Fig. 4 Hydrodynamic voltammogram of catechins: $\circ$, EGC; $\bullet$, GC; $\blacktriangle$, C; $\triangle$, EGCg; $\square$, GCg; $\blacksquare$, EC; $\blacklozenge$, ECg; $\diamond$, Cg. CLC conditions: column, capillary column (150 mm $\times$ 0.2 mm i.d., 3 μ m); column temperature, 50°C; mobile phase, phosphoric acid (85%)-methanol-water (0.5:27.5:72.5, v/v/v); flow rate, 4.0 μ l min⁻¹.

Fig. 5 Chromatogram of catechin standards obtained by CLC-ECD. Each catechin was injected into CLC-ECD in a 25 fmol amount. Peak: 1, GC; 2, EGC; 3, C; 4, EGCg; 5, EC; 6, GCg; 7, ECg; 8, Cg; 9, propyl gallate (I.S.). The CLC-ECD conditions used were the same as in Fig. 4 except for the applied potential, +0.85 V vs. Ag/AgCl.

during separation, was maintained at 50°C. Flow rate was selected at 4.0 μ l min⁻¹. Under these conditions, the chromatographic peaks of GC, EGC, C, EGCg, EC, GCg, ECg, Cg, and propyl gallate (I.S.) were obtained at 1.20, 1.53, 1.71, 2.25, 2.50, 3.03, 4.24, 5.77, and 7.53 min, respectively. The resolution (R_s) of GC and EGC, EGC and C, C and EGCg, EGCg and EC, EC and GCg, GCg and ECg, ECg and Cg, and Cg and I.S. were 2.68, 1.26, 3.15, 1.33, 2.53, 4.71, 4.67, and 8.26, respectively. The relative standard deviations (RSDs) of the retention time for catechins and I.S. peaks were less than 1.9%, indicating that the present CLC-ECD provides accurate separation and elution of catechins.

Thus, the optimal HPLC conditions in this study were: methanol-water-phosphoric acid (27.5:72.5:0.5, v/v/v); flow rate, 4.0 μ l min⁻¹; column temperature, 50°C; and applied potential, +0.85 V vs. Ag/AgCl.

Linear range and detection limit for determining catechins

Figure 5 shows a chromatogram of catechins of 25 fmol, and I.S. The peak height was found to be linearly related to the amount of catechins in the standard solution from 200 amol to 500 fmol ($r > 0.998$), i.e., the concentration of catechins from 4 nM to 10 μ M: from 1.23 ng ml⁻¹ to 3.06 μ g ml⁻¹ (for GC and EGC), 1.16 ng ml⁻¹ to 2.91 μ g ml⁻¹ (for C and EC), 1.83 ng ml⁻¹

to 4.58 μ g ml⁻¹ (for GCg and EGCg), and 1.77 ng ml⁻¹ to 4.42 μ g ml⁻¹ (for Cg and ECg), respectively ($r > 0.998$). The accuracy of the present method is listed in Table 2. The accuracy of the calculated data, as relative error (RE) at 25 fmol, was within the range of -2.8 to 4.4%. In addition, the regression equation with regression coefficients showed good linearity. Each catechin, 25 fmol, was detected with a RSD of 2.2% ($n = 5$). The RSDs of the inter-day precisions for 25 fmol catechins were less than 3.0%. The amount of detection limit ($S/N = 3$) for GC, EGC, C, EGCg, EC, GCg, ECg, and Cg were 54, 61, 61, 75, 75, 67, 75, and 89 amol, respectively, i.e., the concentration of detection limit ($S/N = 3$) for GC, EGC, C, EGCg, EC, GCg, ECg, and Cg were 331, 374, 354, 459, 435, 614, 664, and 787 pg ml⁻¹, respectively. These results do indicate that attomole quantities of catechins can be determined with the presented CLC-ECD system. The detection limits of amount and concentration of catechins by the present CLC-ECD system were compared with those of HPLC-UV,⁵ CLC-UV,¹⁸ HPLC-FL,⁶ HPLC-CL,⁷ HPLC-MS,¹¹ HPLC-ECD with conventional column,^{8,9} and HPLC-ECD with microbore column¹⁰ (Table 3). The comparison of detection limits showed that the present method is the most sensitive technique. The high sensitivity of our method is achieved by using a capillary

Table 3 Detection limit of catechins by various methods

Method	Column size		Injection volume/ μl	Detection limit (catechin)	
	I.D./mm	Length/mm		Amount	Concentration
CLC-ECD	0.2	150	0.05	54 amol	331 pg ml^{-1} (GC)
HPLC-UV ⁵	4.6	100	5	200 pmol	18.0 $\mu\text{g ml}^{-1}$ (ECg)
CLC-UV ¹⁸	0.3	250	0.06	69 fmol	290 ng ml^{-1} (EC)
HPLC-FL ⁶	4.0	250	25	400 fmol	5.00 ng ml^{-1} (C)
HPLC-CL ⁷	4.0	250	50	100 fmol	920 pg ml^{-1} (EGCg)
HPLC-MS ¹¹	4.6	150	10	20 fmol	612 pg ml^{-1} (EGC)
HPLC-ECD ⁸	4.0	250	5	500 fmol	29.0 ng ml^{-1} (GC)
HPLC-ECD ⁹	4.6	250	80	160 fmol	920 pg ml^{-1} (EGCg)
HPLC-ECD ¹⁰	1.0	150	5	5 fmol	310 pg ml^{-1} (GC)

Table 4 Determination of catechins in commercial canned green tea and recovery of catechins from the commercial canned green tea spiked catechins' standard

	Content ($n = 5$)		Rec. ^a ($n = 5$)		
	Conc./ $\mu\text{g ml}^{-1}$	RSD, %	Added conc./ $\mu\text{g ml}^{-1}$	Rec., %	RSD, %
GC	166	0.38	123	97.6	1.2
EGC	52.1	0.87	61.3	97.4	0.85
C	20.4	1.7	29.0	101	2.6
EGCg	84.0	1.6	91.7	102	1.2
EC	11.6	1.6	14.5	99.4	1.4
GCg	55.5	1.5	91.7	100	1.3
ECg	13.3	3.8	22.1	101	0.62
Cg	26.0	1.3	22.1	98.9	1.4

a. For recovery test, catechins standard at each concentration were directly spiked to commercial canned green tea. Catechins derived from both commercial canned green tea and the standard were extracted, and a test solution was injected into CLC-ECD to obtain a chromatogram. The recovery was 100% when the each catechin concentration in the recovery test was equal to the sum of each catechin in commercial canned green tea and the spiked standard.

column that avoids diffusing samples and makes the flow rate slow. Therefore, highly concentrated and undiluted analyte reaches at the surface of working electrode, increasing the electrolytic efficiency of the analyte on the working electrode. Although the concentration sensitivity of the HPLC-ECD with microbore column¹⁰ is almost the same as in the present CLC-ECD, the volume of test solution necessary for one assay in the CLC-ECD is small because sample injection volume is only 50 nl. On the other hand, the sample injection volume in HPLC-ECD with microbore column is 5 μl .¹⁰ The reduction of sample injection volume decreased the use of plasma sample for determining catechins. This lightens a load of a human subject.

Catechins in commercial canned green tea for ingestion were determined qualitatively and quantitatively. Catechin contents in the commercial canned green tea along with catechins' recovery data are listed in Table 4. RSD ($n = 5$) in all cases was less than 3.8%. Catechins' recovery for spiked test solutions was more than 97.4% and RSD ($n = 5$) was less than 2.6%, indicating that the present method provides quite accurate measurements of catechins in commercial canned green tea. We were found that the major catechins in this commercial canned green tea were GC, EGC, EGCg, and GCg.

Determination of catechins in 10- μl plasma samples

Validated data of catechin quantification in a biological

Fig. 6 Chromatograms of catechins obtained from (A) blank and (B) sample plasma of subject T at 1 h after ingestion of 340 ml commercial canned green tea. Peak: 4, EGCg; 5, EC; 6, GCg; 7, ECg; 8, Cg; 9, propyl gallate (I.S.). Each catechin peak corresponded to the total amount of free and conjugated forms. The CLC-ECD conditions used were the same as in Fig. 5.

sample will establish the CLC-ECD usefulness in the biological field. The concentration profiles of free and conjugated form catechins in human plasma were determined before and after commercial canned green tea ingestion for subject T. Figures 6A and B show chromatograms of blank plasma of subject T and plasma sample 1 h after ingestion of 340 ml of commercial canned green tea, respectively. Total concentrations of free and conjugated forms of catechins were determined using 10 μl of human plasma 1 h after ingestion of commercial canned green tea (Table 5). RSD ($n = 5$) was less than 5.0%. The recoveries of catechins were more than 87.5% and RSD ($n = 5$), less than 5.2%, indicating the present method to be quite adequate for monitoring catechins in human plasma.

The time course of concentrations of GCg, EC, EGCg, ECg, and Cg in subject T plasma after ingestion of 340 ml of commercial canned green tea is shown in Fig. 7. Conjugated form concentrations of GCg, EC, EGCg, ECg, and Cg in plasma at 1 h after the ingestion of green tea were 13.7, 18.7, 3.5, 3.2, and 3.5 ng ml^{-1} , respectively. In addition, free-form concentrations of GCg, EC, EGCg, ECg, and Cg in plasma at 1 h after the ingestion of green tea were 3.5, 0, 9.7, 3.2 and 1.3 ng ml^{-1} , respectively. Since there were large peaks at the same retention times on the chromatogram of blank plasma, GC and EGC could not be evaluated. The C concentration was less than the detection limit ($<354 \text{ pg ml}^{-1}$). GCg, EC, EGCg, ECg, and Cg concentrations began to increase following green tea ingestion; each conjugated and free forms of catechins reached a maximum concentration in plasma at about 1–2 h after. GCg

Table 5 Determination of total concentration of free and conjugated forms of catechins in human plasma at 1 h after the ingestion of commercial canned green tea and recovery of catechins from the plasma spiked catechins' standard

	Content ($n = 5$)		Recovery ^a ($n = 5$)		
	Conc./ $\mu\text{g ml}^{-1}$	RSD, %	Added conc./ $\mu\text{g ml}^{-1}$	Rec., %	RSD, %
GCg	17.2	5.0	22.9	91.4	5.2
EC	18.8	2.6	14.5	94.2	1.8
EGCg	13.2	1.2	22.9	87.5	2.0
ECg	6.30	4.5	22.1	89.5	4.2
Cg	4.76	3.1	22.1	92.7	2.6

a. For recovery test, catechins standard at each concentration were directly spiked to human plasma at 1 h after the ingestion of commercial canned green tea. Catechins derived from both human plasma and the standard were extracted, and a test solution was injected into CLC-ECD to obtain a chromatogram. The recovery was 100% when the each catechin concentration in the recovery test was equal to the sum of each catechin in human plasma and the spiked standard.

and EGCg concentrations in plasma were higher than ECg and Cg. This tendency was similar to the concentrations of catechins in commercial canned green tea. The conjugated form of EC in plasma attached at 20.0 ng ml^{-1} , although the EC concentration in the commercial canned green tea was relatively low. In regard to EC, Cg, and GCg in plasma, their conjugated forms were more abundant than their free forms. But, for EGCg and ECg in plasma, their free forms were at higher levels than their conjugated forms.

In our previous study, we determined catechins in human plasma by HPLC-ECD with a microbore column.¹⁰ Because sample injection volume in the HPLC-ECD with microbore column was $5 \mu\text{l}$, at least $50 \mu\text{l}$ of test solution before filtration by $0.45 \mu\text{m}$ membrane filter was practically needed. Therefore, $100 \mu\text{l}$ plasma sample was needed to prepare a test solution for determining catechin concentration. In the case of human subject, to prepare $100 \mu\text{l}$ plasma, more than $200 \mu\text{l}$ blood specimens must be collected from vein, and the measurable point numbers for determining time course of plasma catechins concentrations were limited. On the other hand, because sample injection volume in the present CLC-ECD is only 50 nl , the test solution before filtration by $0.45 \mu\text{m}$ membrane filter needs to be at least $10 \mu\text{l}$. Therefore, because only $10 \mu\text{l}$ plasma sample is needed to prepare a test solution for determining catechin concentration, blood specimen are obtained using a lancing device from a finger by oneself and/or an analyst who is not a medical doctor, a nurse, and a medical technologist, and determination of catechins in human plasma after ingestion of commercial canned green tea were carried out by the present method. The present CLC-ECD using small volume of blood specimen has the potential to determine detailed time course of plasma catechins concentration based on a data set of many point numbers in a subject, and it would be applied to elucidate their actual and detailed kinetic metabolisms of catechins.

Moreover, because the measurement time and flow rate in the present CLC-ECD are 10 min and $4.0 \mu\text{l min}^{-1}$, respectively, the waste volume for determining catechins in one assay is decreased by about a fiftieth compared with the HPLC-ECD with microbore column. This is very profitable from the viewpoint of green chemistry.

Fig. 7 Time course of catechins concentrations of (A) conjugated form and (B) free form in plasma after ingestion of 340 ml commercial canned green tea. Catechin: □, GCg; ■, EC; △, EGCg; ◆, ECg; ◇, Cg.

Conclusion

The CLC-ECD using an isocratic elution system was developed for determining attomole quantities of catechins within 10 min. The capillary column with ECD increases sensitivity though a decrease in band broadening that results in sharper peaks. Therefore, the CLC-ECD method presented is the most sensitive one for determining catechins when compared to other methods. Furthermore, the reduced scale by which CLC needs lower amounts of mobile phases. From the point of view of environmentally friendly chemistry, CLC has the advantage of requiring smaller amounts of waste liquids over conventional HPLC.

Moreover, the presented CLC-ECD was applied for determining catechins in human plasma after ingestion of green tea. Catechins in only $10 \mu\text{l}$ human plasma which was obtained by a lancing device were determined with good precision by the present method. It is suitable from the viewpoint of bioethics to reduce the burden for a human subject. The present method will also be useful for further investigation of the metabolism of catechins in mammals.

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