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Journal of Chromatography B, 788 (2003) 269–275
JOURNAL OF CHROMATOGRAPHY B
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Determination of catechins in human plasma after commercial canned green tea ingestion by high-performance liquid chromatography with electrochemical detection using a microbore column

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Received 24 June 2002; received in revised form 2 December 2002; accepted 20 December 2002

Abstract

Determination of catechins in human plasma was carried out by high-performance liquid chromatography with electrochemical detection using a microbore octadecylsilica column. Peak heights for catechins were found to be linearly related to the amount of each catechin injected, from 2 pmol/ml to 2 nmol/ml ($r>0.999$). Conjugated-form catechins in plasma were hydrolyzed enzymatically using β-glucuronidase and sulfatase. Catechins in plasma and the hydrolyzed solution were extracted with ethyl acetate and determined by the present method. The time courses of concentrations of catechins in human plasma showed maxima at 1–2 h after ingestion of 340 ml of commercial canned green tea.

Keywords: Catechins

1. Introduction

Catechins [catechin (C), epicatechin (EC), gallocatechin (GC), epigallocatechin (EGC), catechin gallate (Cg), epicatechin gallate (ECg), gallocatechin gallate (GCg), and epigallocatechin gallate (EGCg), Fig. 1] in green tea are of interest for potential application to cancer and coronary heart disease prevention. These compounds have been found to exert antioxidative [1], antitumoral [2], and antimutagenic effects [3]. HPLC with UV detection (HPLC–UV) and HPLC with electrochemical detection (HPLC–ED), capillary electrophoresis (CE) are used at present for determining catechins [4–6] in tea, rat plasma [7], human plasma [8–12], and urine [6]. Gas chromatography with mass spectrometry (GC–MS) [13] is a highly sensitive method for determination of catechins. HPLC, on the other hand, is widely available.

To elucidate the kinetic metabolism of these compounds in the human body, research has been carried out at relatively high concentration of catechins [12] since a sensitive analytical method was not available. In order to discuss the effects of catechins on the human body after ingestion of daily food and tea, a methodology with high sensitivity is necessary

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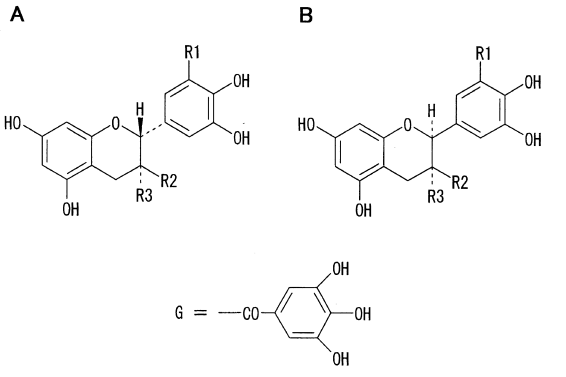
1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(02)01036-X



A	R1	R2	R3	B	R1	R2	R3
(−)-EC	H	H	OH	(−)-C	H	H	OH
(−)-ECg	H	H	OG	(−)-Cg	H	H	OG
(−)-EGC	OH	H	OH	(−)-GC	OH	H	OH
(−)-EGCg	OH	H	OG	(−)-GCg	OH	H	OG

Fig. 1. Structures of the catechins.

for determining catechins in human plasma. Highly sensitive analytical methods will help to clarify the effects of various catechins on the mechanisms of disease.

In the present study, an HPLC–ED method using a microbore octadecylsilica (ODS) column was developed for determining catechins. The HPLC–ED system with its high sensitivity, was applied to the quantification of five catechins in human plasma subsequent to the ingestion of one can of commercial green tea.

2. Experimental

2.1. Reagents

(−)-Gallocatechin (>98%), (−)-epigallocatechin (>98%), (−)-catechin (>98%), (−)-epigallocatechin gallate (>98%), (−)-epicatechin (>98%), (−)-gallocatechin gallate (>98%), (−)-epicatechin gallate (>98%), and (−)-catechin gallate (>98%) were obtained from Kurita Industrial (Tokyo, Japan). Other reagents and HPLC grade solvents were from Wako (Osaka, Japan). Commercial canned green tea samples were purchased from Kirin Beverage. β-Glucuronidase (G-7896) and sulfatase (S-9754) were obtained from Sigma.

2.2. Apparatus and HPLC–ED conditions

The HPLC–ED equipment with a microbore column comprised of a LC-26A vacuum degasser (BAS, Tokyo, Japan), LC-100 pump (BAS), 7125 injector fitted with a 5-μl injection loop (Reodyne, Cotati, CA, USA), Capcell Pak C_{18} UG 120 microbore ODS column (150 mm×1.0 mm I.D., 3 μm, Shiseido, Tokyo, Japan), FT-1 column oven (BAS), and a LC-4C electrochemical detector (BAS). The commercially available electrochemical cell (Radial flow cell, BAS) was constructed from a glassy carbon working electrode, an Ag/AgCl reference electrode, and a stainless steal auxiliary electrode. A 5-μl sample solution or standard catechins solution was injected into the microbore ODS column maintained at 40 °C. A deaerated phosphoric acid (85%)–water–methanol (0.5:81:19, v/v/v) solution served as the mobile phase, at a flow-rate of 25 μl/min. The detection potential for monitoring catechins was set at +0.6 V vs. Ag/AgCl. An internal standard (I.S.) method was used for determination of each catechin concentration in the sample solution, and ethyl gallate was used as the I.S.

2.3. Preparation of tea sample

In order to prepare a test solution, a sample of commercial canned green tea (10 ml) was mixed with an equivalent amount of phosphoric acid (85%)–water–methanol (0.5:81:19, v/v/v) mixture and sonicated for 1 min, diluted 500 times again with the above mixture, and then filtered through a 0.45-μm membrane filter. A 5-μl volume of the test solution was injected into the HPLC system.

2.4. Sample preparation of free- and conjugated-form catechins in plasma

All plasma samples were obtained from two healthy male volunteers (subject K, 28 years old, 60 kg body weight, nonsmoker and subject M, 22 years old, 65 kg body weight, nonsmoker), who were not permitted to take 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. Plasma samples were taken before tea

ingestion and 1, 1.5, 2, 2.5, 3, 4, and 6 h after ingestion from subject K, and 1, 2, 4 and 6 h after ingestion from subject M, respectively. All plasma samples were stored at −30 °C until analysis.

A 100-μl volume of plasma was mixed with 10 μl 0.4 *M* phosphate buffer (pH 3.6) containing 2% ascorbic acid and 0.1% ethylenediaminetetraacetic acid disodium salt, and 30 μl 0.1 *M* phosphate buffer (pH 6.8) containing 500 U β-glucuronidase, 40 U sulfatase, and 0.5 μ*M* ethyl gallate as the internal standard. The mixture was incubated at 37 °C for 45 min. Catechins in the enzyme-hydrolyzed solution were extracted three times with 200 μl of aliquots of ethyl acetate. The collected ethyl acetate was evaporated to dryness using a nitrogen stream. The dry residue was dissolved in a phosphoric acid (85%)–water–methanol (0.5:81:19, v/v/v) solution. The solution was filtered through a 0.45-μm membrane filter 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: 100 μl of plasma were mixed with the above-mentioned solution without enzyme, catechins in the mixture were extracted with ethyl acetate, and the collected ethyl acetate was evaporated to dryness using a nitrogen stream. A 5-μl volume of test solution was injected into the HPLC–ED system.

3. Results and discussion

3.1. Catechin determination using the microbore column

Electrochemical oxidation of catechins has been studied by many researchers [14–16]. Yang et al. [16] discussed the structural effects on the oxidation potential in methanol–0.1 *M* phosphate buffer (pH 7.5) (1:1, v/v) for (−)-GC, (−)-EGC, (−)-C, (−)-EGCg, (−)-EC, (−)-GCg, (−)-ECg, and (−)-Cg. (−)-GC and (−)-EGC, having an *ortho*-trihydroxyl group on the B-ring, showed the lowest half-wave potential ($E_{1/2}$) values. (−)-C and (−)-EC, containing an *ortho*-dihydroxyl group on the B-ring, possessed the highest $E_{1/2}$ values. Thus, $E_{1/2}$ of pyrogallol-containing catechins were lower than that of catechol-containing catechins.

To determine the detection potentials for eight catechins, hydrodynamic voltammograms were obtained using a mobile phase of phosphoric acid (85%)–water–ethanol (0.5:81:19, v/v/v) and an electrochemical detector. As shown in Fig. 2, two-step oxidation waves were noted for all catechins examined. At a detection potential more positive than +1.0 V vs. Ag/AgCl, the sensitivity was relatively high, but reproducibility of peak height was poor. Therefore, the detection potential for catechins was selected at +0.6 V vs. Ag/AgCl.

Examination was made of how the ratio of water to methanol in the mobile phase influenced the separation for determination of catechins. The larger the content of water, the greater was the separation of the catechins' peaks. The column temperature also influenced the separation: the lower the column temperature, the greater was the separation of the catechins' peaks within 120 min. To determine catechins in tea and plasma with adequate resolution within a short time, water–methanol (81:19, v/v) was chosen for the most suitable mobile phase and the column temperature during the separation was maintained at 40 °C.

Fig. 3 presents a chromatogram of a standard mixture containing 0.2 nmol/ml of each of the following: (−)-GC, (−)-EGC, (−)-C, (−)-EGCg, (−)-EC, ethyl gallate (I.S.), (−)-GCg, (−)-ECg, and

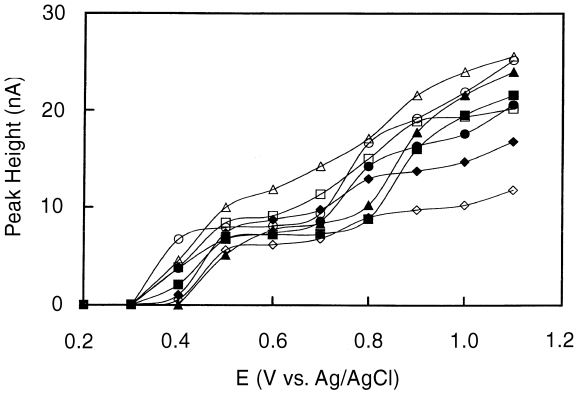


Fig. 2. Hydrodynamic voltammogram of catechins. Catechins: △, (−)-EGCg; ○, (−)-EGC; ▲, (−)-C; ■, (−)-EC; ●, (−)-GC; □, (−)-GCg; ♦, (−)-ECg; ◇, (−)-Cg). HPLC conditions: column, microbore ODS column (150 mm×1.0 mm I.D., 3 μm); column temperature, 40 °C; mobile phase, phosphoric acid (85%)–water–ethanol (0.5:81:19, v/v/v); flow-rate, 25 μl/min.

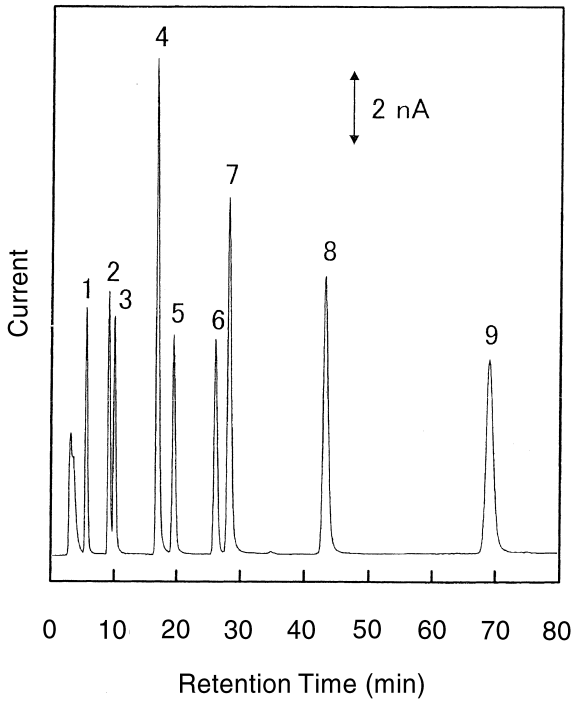


Fig. 3. Chromatogram of standard catechin obtained by HPLC–ED with microbore column. Each catechins was injected into HPLC in concentration of 0.2 nmol/ml. Peaks: 1, (−)-GC; 2, (−)-EGC; 3, (−)-C; 4, (−)-EGCg; 5, (−)-EC; 6, ethyl gallate (internal standard); 7, (−)-GCg; 8, (−)-ECg; 9, (−)-Cg. HPLC conditions: column, microbore ODS column (150 mm×1.0 mm I.D., 3 μm); column temperature, 40 °C; mobile phase, phosphoric acid (85%)–water–ethanol (0.5:81:19, v/v/v); flow-rate, 25 μl/min; applied potential, +0.6 V vs. Ag/AgCl.

(−)-Cg. Retention times of (−)-GC, (−)-EGC, (−)-C, (−)-EGCg, (−)-EC, (−)-GCg, (−)-ECg, and (−)-Cg were 5.8, 9.2, 10.4, 16.9, 19.6, 26.5, 28.5, 43.5, and 70.0 min, respectively. Resolutions (R_s) of (−)-GC and (−)-EGC, (−)-EGC and (−)-C, (−)-C and (−)-EGCg, (−)-EGCg and (−)-EC, (−)-EC and ethyl gallate, ethyl gallate and (−)-GCg, (−)-GCg and (−)-ECg, and (−)-ECg and (−)-Cg were 5.31, 1.77, 7.99, 2.90, 10.6, 1.97, 9.55 and 13.4, respectively. Peak height was found to be linearly related to the concentration of the respective injected catechin in the standard mixture solution, from 2 pmol/ml to 2 nmol/ml: from 920 pg/ml to 920 ng/ml [against (−)-GCg and (−)-EGCg], 890 pg/ml to 890 ng/ml [against (−)-Cg and (−)-ECg], 610 pg/ml to 610 ng/ml [against (−)-GC and (−)-EGC], and 580 pg/ml to 580 ng/ml [against (−)-C and (−)-EC], respectively ($r>0.999$). Standard solutions of (−)-GC at 30.6 ng/ml, (−)-EGC at 30.6 ng/ml, (−)-C at 29.1 ng/ml, (−)-EGCg at 45.8 ng/ml, (−)-EC at 29.1 ng/ml, (−)-GCg at 45.8 ng/ml, (−)-ECg at 44.2 ng/ml, and (−)-Cg at 44.2 ng/ml were determined with relative standard deviation (RSD, $n=10$) values of 0.54, 0.44, 0.43, 0.50, 0.36, 0.52, 0.58, and 0.58, respectively. The detection limits ($S/N=3$) of the present method for (−)-GCg, (−)-EGCg, (−)-Cg, (−)-ECg, (−)-GC, (−)-EGC, (−)-C, and (−)-EC were 460, 460, 440, 440, 310, 310, 290 and 290 pg/ml, respectively. Detection limits of catechins by the present method were compared with those of HPLC–UV [5], HPLC–ED with a conventional column [6,8], HPLC with fluorescence detection (HPLC–FL) [9], HPLC with chemiluminescence detection (HPLC–CL) [11], CE [4], and GC–MS [13] as shown in Table 1. The present HPLC–ED was more sensitive than the other methods, except GC–MS. Although, GC–MS is more sensitive than the present method, Soleas et al. could determine only C of the catechins investigated in the current study [13], whereas using the present HPLC–ED method, eight separate catechins could be determined in one assay.

3.2. Determination of catechins in plasma subsequent to commercial canned green tea ingestion

Catechins in commercial canned green tea were determined qualitatively and quantitatively. The content of catechins in the commercial canned green tea along with their recovery data are listed in Table 2. RSD ($n=5$) value in all cases was less than 3.9%. Catechins' recovery for spiked test solutions was >98% and RSD ($n=5$) was <2.5%, indicating 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.

The concentration profile of free and conjugated form catechins in plasma was determined before and after commercial canned green tea ingestion for subjects K and M. Fig. 4A–C shows chromatograms of blank plasma of subject K, 1 h after ingestion of

Table 1
Detection limit of catechins by various methods

Method	Column size		Detection limit (catechins)	Injection volume (μl)
	I.D. (mm)	Length (mm)		
HPLC–ED with microbore column	1.0	150	290 pg/ml (Cg)	5
HPLC–UV [5]	4.6	100	18.0 μg/ml (ECg)	5
HPLC–ED [6]	4.0	250	29.0 ng/ml (Cg)	5
HPLC–ED [8]	4.6	250	920 pg/ml (EGCg)	80
HPLC–FL [9]	4.0	250	5.00 ng/ml (C)	25
HPLC–CL [11]	4.0	250	920 pg/ml (EGCg)	50
CE [4]			50 ng/ml (C)	
GC–MS [13]			10 pg/ml (C)	1

340 ml of commercial canned green tea, and spiked 58–91 ng/ml standard catechins to plasma sample 1 h after the ingestion of 340 ml of the commercial canned green tea, respectively. To determine the recovery of each catechin during sample preparation, 58–91 ng/ml standard solution of catechins were added to 100 μl plasma sample 1 h after ingestion of green tea, and the mixture was incubated and analyzed as described. The recoveries of GCg, EC, EGCg, ECg, and Cg in plasma were 89.6, 96.3, 95.8, 92.1 and 97.1%, respectively.

The time courses of concentrations of GCg, EC, EGCg, ECg, and Cg in subject K plasma after ingestion of 340 ml of commercial canned green tea are shown in Fig. 5A. Conjugated form concentrations of GCg, EC, EGCg, ECg, and Cg in plasma at 1 h after the ingestion of green tea were 51.2, 11.5, 16.5, 5.1 and 5.8 ng/ml, 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 4.9, 0, 17.1, 5.8 and 0 ng/ml, 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 (<290 pg/ml). GCg, EC, EGCg, ECg, and Cg concentrations began to increase following green tea ingestion, GCg, EC, EGCg, ECg, and Cg reached a maximum concentration in plasma at 1 h. GCg and EGCg concentrations in plasma were higher than ECg and Cg. This tendency was similar to the concentration of catechins in commercial canned green tea. EC in plasma attained at 11.5 ng/ml, although EC concentration in the commercial canned green tea was relatively low. The conjugated forms of EC, Cg, and GCg in plasma were more abundant

Table 2
Contents of catechins in commercial canned green tea and recovery of catechins from commercial canned green tea spiked with catechins' standards

Catechins	Content ($n=5$)		Recovery ($n=5$)		
	Concentration (μg/ml)	RSD (%)	Concentration added (μg/ml)	Recovery (%)	RSD (%)
GC	127	0.27	123	100	1.1
EGC	63.4	0.89	61.3	99	0.82
C	33.0	1.5	29.0	104	2.5
EGCg	94.0	1.4	91.7	101	1.1
EC	15.4	1.2	14.5	100	1.3
GCg	89.5	1.3	91.7	101	1.1
ECg	18.5	3.9	22.1	102	0.61
Cg	20.6	1.2	22.1	98	1.2

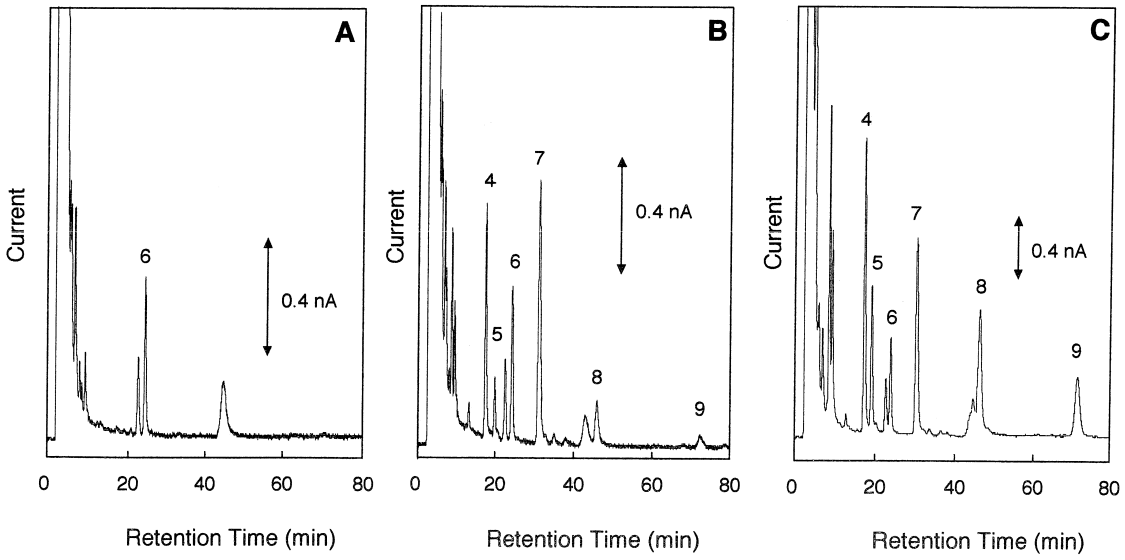


Fig. 4. Chromatogram of catechins obtained from (A) blank and (B) sample plasma of subject K at 1 h after ingestion of 340 ml commercial canned green tea. (C) Spiked standard catechins in sample plasma of subject K at 1 h after ingestion of 340 ml commercial green tea. Peaks: 4, EGCg; 5, EC; 6, ethyl gallate (I.S.); 7, GCg; 8, ECg; 9, Cg. Each catechin peak corresponded to the total amount of free and conjugated forms. HPLC conditions as in Fig. 3.

than their free forms, but for EGCg and ECg in plasma, their free forms were at higher levels than their conjugated forms.

In the case of subject M, the time course of concentrations of GCg, EC, EGCg, ECg and Cg in subject M plasma after ingestion of 340 ml of

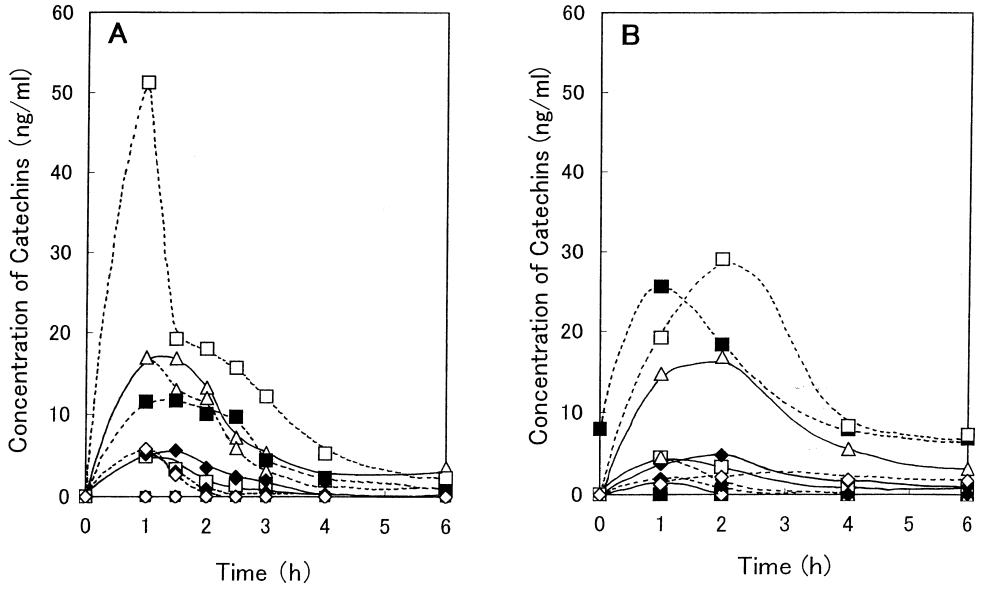


Fig. 5. Time course of plasma of (A) subject K (male, 28 year old) and (B) subject M (male, 22 year old) catechins of conjugated form (· · ·) and free form (———). Catechins: □, GCg; ■, EC; △, EGCg; ♦, ECg; ◇, Cg.

commercial canned green tea are shown in Fig. 5B. EC was detected in blank plasma and after green tea ingestion of subject M. The conjugated form concentration of EC in blank plasma was 7.98 ng/ml, and free form of EC in blank plasma was not detected. This EC in subject M blank plasma may be derived from his diet as no one has reported the presence of EC in blank plasma [12]. The conjugated-form concentrations of GCg, EC, EGCg, ECg, and Cg in plasma at 1 h after the ingestion of green tea were 19.19, 25.58, 4.54, 1.89 and 1.26 ng/ml, 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 4.49, 0, 14.74, 3.77 and 1.18 ng/ml, respectively. These results were similar to the time course results of the concentrations of catechins in the plasma of subject K.

In our previous study, we determined catechins in human urine by conventional HPLC–ED [6]. For both GC and EGC, the conjugated form, showed a maximum excretion at 1–3 h after ingestion. After 10 h, the free form increased to become the main form. For C and EC, the maximum excretion of the conjugated form was found at 2–3 h after ingestion. The free form was detected, too. GCg, EGCg, ECg and Cg were not contained in urine after commercial canned ingestion green tea. But, this conventional HPLC–ED method was unable to determine catechins in human plasma, because conventional HPLC–ED has a lower sensitivity than the presented HPLC–ED using a microbore column.

4. Conclusion

HPLC–ED with a microbore column was developed for determining catechins in human plasma and in commercial canned green tea. Because the diffusion of the injected sample in the system for HPLC with a microbore column seemed to be less than that for HPLC with a conventional column, the sensitivity by the former method is superior to the latter. Therefore, catechins in only 100 μl human plasma after ingestion of commercial canned green tea, a natural beverage which has a relatively lower concentration of catechins than in green tea leaf extraction, were determined by the present method. The present method will also be useful for further investigation of the metabolism of catechins in the human body.

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