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Simultaneous determination of catechins in human saliva by high-performance liquid chromatography

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Abstract

Green tea extracts have been suggested to possess a preventive effect against dental caries. A quantitative method for their anticariogenic substances, catechins, was developed to evaluate their concentrations in human saliva after mouthrinsing with green tea extract. Salivary catechins were extracted to the organic phase after forming a complex with diphenylborate and an ion-pair with tetra-*n*-butylammonium, and then back-extracted to the acidic aqueous phase. The extract was analyzed by high-performance liquid chromatography using diode array detection at absorption wavelengths ranging from 269 to 278 nm. In reversed-phase chromatography by a gradient elution, eight catechins originating from green tea and an internal standard were separated in 15 min without interfering peaks. All the catechins were simultaneously and selectively determined in the concentration range 0.05–25.0 μg/ml. In replicate spiking experiments with standards, the mean recovery ranged between 86 and 99%, and both intra- and inter-assay C.V.s were within 2.3%. When mouthrinsing with an aqueous solution of green tea extract (5.0 mg/ml) containing eight catechins, the quantitative results revealed that each catechin was retained at μg/ml levels in saliva for up to 60 min. © 1997 Elsevier Science B.V.

Keywords: Saliva; Tea; Catechins

1. Introduction

Catechins originating from tea leaves (*Camellia sinensis*) have been suggested to possess various pharmacological activities [1–6]. Among them, the anticariogenicity of catechins has been practically utilized for the prevention of dental caries [7]. In Japan, green tea extracts containing catechins have been widely added to candy, chewing gum, food and mouthrinsing agents as a caries preventive additive. The effect of catechins is attributed to their antibacterial activity against oral cariogenic bacteria [8], and to their inhibitory activity against dental plaque formation and bacterial adherence to the tooth surface [9,10]. When tea extracts are applied to the oral cavity, the presence of catechins in active concentrations in saliva is the determinant for their potent anticariogenic activity; therefore the concentrations of salivary catechins have interested researchers.

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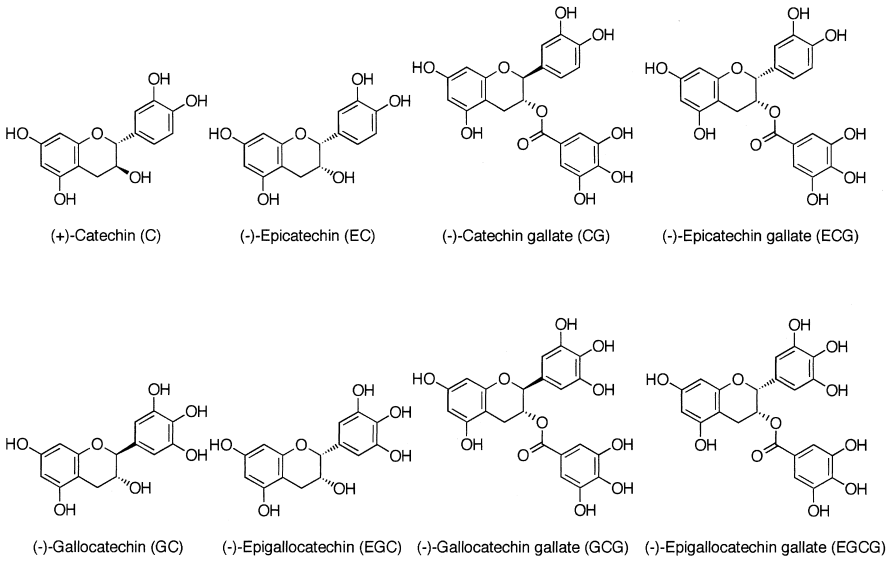

Fig. 1. Structures of tea catechins.

Since tea catechins are composed of eight catechins with different substituents (Fig. 1), a chromatographic technique is suitable for their separative analysis. Although several high-performance liquid chromatography (HPLC) methods were previously reported for catechin analysis, they were specific for only some of the catechins in beverage and blood samples, but could not achieve the simultaneous separation of all eight catechins [11–14]. Another methodological problem is the low specificity to catechins as previous methods lacked a selective purification pretreatment [11,12,14,15].

The specific complexing reaction of borate with 1,2-diols has been used for purifying catecholamines and catechol amino acids from biological samples [16–18]. All the catechins in green tea are polyhydroxyflavan derivatives that possess the 1,2-dihydroxyl structure in the B ring as well as catechol compounds (Fig. 1). Considering this common structure, catechins are also presumed to be selectively extractable from saliva by forming a complex with diphenylborate.

In the present study, we developed an HPLC procedure for the simultaneous analysis of all eight catechins using borate-complex extraction as a purification pretreatment. Salivary catechins were determined to evaluate their concentrations in human saliva after mouthrinsing with green tea extract.

2. Experimental

2.1. Reagents and chemicals

(+)-Catechin (C), (−)-epicatechin (EC), (−)-gallocatechin (GC), (−)-epigallocatechin (EGC), (−)-catechin gallate (CG), (−)-epicatechin gallate (ECG), (−)-gallocatechin gallate (GCG) and (−)-epigallocatechin gallate (EGCG) were purchased from Funakoshi (Tokyo, Japan). The purities of all standard catechins were more than 98% based on HPLC analysis. Their stock solutions (1.0 mg/ml of each) were prepared by dissolving the standards in 0.01 *M* HCl, and they were then stored at 4°C. Under these conditions, catechins were stable for at least 1 month. They were diluted as required with 0.01 *M* HCl before use. 4-Methylcatechol (4-MC), diphenylborate–ethanolamine complex and tetra-*n*-butylammonium bromide of ion-pair chromatographic grade were obtained from Tokyo Kasei (Tokyo, Japan), Aldrich (Milwaukee, WI, USA) and Nacalai Tesque (Kyoto, Japan), respectively. Green tea ex-

tract, Sunphenon, was manufactured by Taiyo Kagaku (Yokkaichi, Japan). All other reagents were of the highest analytical grade available. Water was redistilled by an all-glass apparatus after purification by a Milli-Q water purification system (Nihon, Millipore, Tokyo, Japan).

2.2. Saliva samples

Unstimulated whole saliva was collected from five male subjects, aged 43 to 45 years, according to the standard guidelines for saliva collection [19]. All subjects had not consumed tea or tea-related beverages for at least 12 h prior to the experiment. The subjects rinsed their mouths with an aqueous solution of green tea extract (5.0 mg/ml). Each mouthrinsing consisted of five consecutive 30 s rinsings with 20 ml of the solution (total volume 100 ml). They expectorated saliva after the final mouth rinse. Saliva (0.5 ml) was collected before mouthrinsing and at 1, 10, 30 and 60 min intervals after mouthrinsing. Immediately after collection, the saliva was diluted 5- or 10-fold with 0.01 *M* HCl, followed by the extraction procedure.

2.3. Sample preparation

Extraction procedures were performed using polypropylene tubes with screw caps (101×14 mm I.D., Sarstedt, Nümbrecht, Germany). To 0.5 ml of each saliva diluent were added 50 μl of a 4-MC solution (1.0 mg/ml) in 0.01 *M* HCl used as an internal standard, 0.5 ml of 0.15% (w/v) diphenylborate in 5% (v/v) ethanol–2.0 *M* potassium phosphate buffer (pH 8.5) and 4.0 ml of 5 m*M* tetra-*n*-butylammonium bromide in 20% (v/v) *n*-octanol–*n*-hexane. The mixture was vigorously shaken for 1 min, and then centrifuged (1200 *g*, 3 min). The supernatant was vigorously shaken with 0.25 ml of 7.5% (v/v) trifluoroacetic acid (TFA) for 1 min. After centrifugation (1200 *g*, 3 min) a 40–100 μl aliquot of the aqueous phase was subjected to HPLC separation.

2.4. HPLC analysis

The HPLC system consisted of an LC-9A liquid chromatograph (Shimadzu, Kyoto, Japan) connected to a KMT-30A autosampler (Kyowa Seimitsu, Tokyo, Japan), a Shim-pack CLC-C8 (M) column (250×4.6 mm I.D., particle size 5 μm, Shimadzu) placed in a thermo controller (Kyowa Seimitsu) and an SPD-M10AVP diode array detection system (Shimadzu) controlled by an FMV-5133D5 personal computer (Fujitsu, Tokyo, Japan). Chromatographic separation was performed by delivering the mobile phase, A: acetonitrile–TFA–water (10:0.3:89.7, v/v/v) and B: acetonitrile–water (30:70, v/v) at a flow-rate of 1.0 ml/min and at a column temperature of 50°C. The gradient was prepared as follows: 100% A at 0 min to 25% A and 75% B at 17 min in a linear gradient elution mode, and then 100% A for 10 min to equilibrate the column for the next run. In routine analyses, the eluates were detected at absorption wavelengths of 269, 274, 277 and 278 nm. The catechins were determined based on the calibration graphs prepared as described in Section 2.5.

2.5. Analytical evaluation

To evaluate the quantitative range, calibration graphs were prepared by plotting peak area ratios of each catechin to 4-MC against the known concentrations. Eight standard catechins (0.025–25.0 μg/ml of each) were added to saliva samples, and the spiked solutions were treated as described in Sections 2.3 and 2.4. The mean ratios of duplicate experiments were plotted.

To evaluate the recovery and analytical precision, eight standard catechins (5.00 μg/ml of each) were added to 5- and 10-fold saliva diluents. Replicate spiked solutions were analyzed as described in Sections 2.3 and 2.4. The mean recovery ($n=8$), and intra- ($n=8$) and inter-assay C.V. ($n=4$) were determined.

3. Results and discussion

Diode array spectra obtained from the standard catechins (5.00 μg/ml of each, chromatographed) showed absorption maxima as follows: 269 nm for GC and EGC, 278 nm for C, CG and EC, 274 nm for EGCG and GCG, 277 nm for ECG and 282 nm for 4-MC. Since the optimal absorption wavelengths varied among analytes, they were detected at 269, 274, 277 and 278 nm in routine analyses. The mean

relative peak areas ($n=4$) of representative catechins at 269, 274, 277 and 278 nm were as follows: 1.00, 0.92, 0.81 and 0.74 for EGC, 0.71, 0.93, 0.98 and 1.00 for C, 0.91, 1.00, 0.96 and 0.95 for EGCG and 0.84, 0.96, 1.00 and 0.97 for ECG, respectively. After optimization of the chromatographic conditions, the reversed-phase system using a C_8 column and a gradient elution achieved the simultaneous separation of eight catechins and 4-MC within 15 min as shown in Fig. 2. Peak tailing effects probably due to an interaction of polyhydroxyl groups of catechins with the stationary phase were prevented by addition of TFA to the mobile phase.

Successful analysis of body fluids primarily depends on performance of the pretreatment as specific to the analytes as possible. The complexing reaction of the 1,2-dihydroxyl structure with certain ligands was recently reported to be suitable for the selective purification of catechins [13]. In that report, alumina was used as a complexing ligand in solid-phase. Since the pretreatment using alumina generally shows a relatively lower recovery and is a cumbersome procedure [20], liquid–liquid extraction using diphenylborate was employed in the present study.

In the extraction step, catechins are considered to specifically react with diphenylborate to form the complex. Since the formed complex is negatively charged [16], it is efficiently extracted to the organic phase by forming an ion-pair with an anionic counter-ion, tetra-*n*-butylammonium. In the back-extraction step, the complex is easily dissociated in acidic conditions [16,17] and catechins in free form transfer to the aqueous phase of a TFA solution.

Catechins are too unstable in alkaline media to decompose oxidatively, although they are relatively stable in acidic and neutral conditions [13,21]. In this study, for selective extraction, salivary catechins were subjected to a complexing reaction with diphenylborate at pH 8.5. Since catechins are stabilized by forming the complex, their decomposition is negligible during analysis [21].

The representative chromatographic results obtained from saliva before and after mouthrinsing with green tea extract are shown in Fig. 2. Salivary catechins were identified by comparing retention times and diode array spectra of their peaks to standards. All analytes, eight catechins and 4-MC showed a baseline separation without major interfering peaks, indicating that the diphenylborate-complex extraction is highly selective for salivary catech-

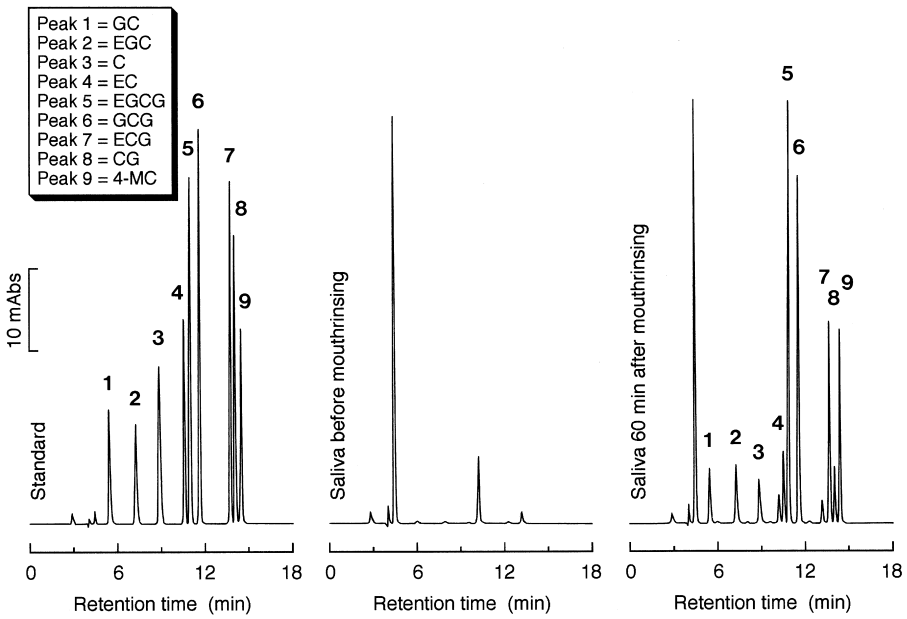

Fig. 2. HPLC chromatograms obtained from standard and saliva samples. Standard catechins (5.00 μg/ml of each), saliva before mouthrinsing, and saliva 60 min after mouthrinsing with green tea extract (5.0 mg/ml). Detection at 274 nm.

Table 1
Recovery and analytical precision

	Saliva 10-fold diluent with 0.01 *M* HCl			Saliva 5-fold diluent with 0.01 *M* HCl		
	Recovery (%)	Intra-assay C.V. (%)	Inter-assay C.V. (%)	Recovery (%)	Intra-assay C.V. (%)	Inter-assay C.V. (%)
GC	97.4	1.2	1.7	96.5	1.4	1.2
EGC	96.4	1.3	1.6	94.3	1.6	1.7
C	98.4	1.3	1.7	96.5	1.3	1.6
EC	98.5	1.3	1.7	97.2	1.0	0.8
EGCG	95.4	1.4	1.9	85.7	1.3	1.6
GCG	96.1	1.7	2.3	89.0	1.2	1.5
ECG	95.5	1.5	2.0	87.9	1.4	1.7
CG	95.9	1.6	2.2	90.0	1.2	1.3

Mean recovery and reproducibility were evaluated by analyzing replicate saliva samples spiked with standard catechins (5.00 μg/ml of each). $n=8$ For recovery, $n=8$ for intra-assay precision and $n=4$ for inter-assay precision.

ins. No catechins were detected in saliva before mouthrinsing with green tea because catechins are not normally the endogenous substances in human body fluids, but exogenously supplied as bioflavonoids through beverage and food ingestion [22,23].

Calibration graphs were linear in the concentration range 0.25–25.0 μg/ml for GC and EGC, 0.10–25.0 μg/ml for C and 0.05–25.0 μg/ml for EC, EGCG, GCG, ECG and CG. The regression equations were found to be $y=0.0863x+0.0156$ ($r^2=0.9985$) for GC, $y=0.081x+0.0135$ ($r^2=0.9982$) for EGC, $y=0.1734x+0.0269$ ($r^2=0.999$) for C, $y=0.1886x+0.0293$ ($r^2=0.999$) for EC, $y=0.3654x+0.0207$ ($r^2=0.9994$) for EGCG, $y=0.4239x+0.0443$ ($r^2=0.9992$) for GCG, $y=0.3814x+0.0362$ ($r^2=0.9993$) for ECG and $y=0.3299x+0.0168$ ($r^2=0.9995$) for CG. These quantitative ranges are high enough to determine salivary catechins of anticariogenic concentrations.

Recovery and analytical reproducibility were evaluated by analyzing replicate spiked saliva samples either on the same day (intra-assay) or on different days (inter-assay). The saliva samples were added with standard catechins which almost correspond to the concentrations in saliva diluents after mouthrinsing with green tea extract. The mean recovery ($n=8$) through all procedures, and the intra- ($n=8$) and inter-assay C.V. ($n=4$) are shown in Table 1.

Salivary catechins were determined after mouthrinsing with green tea extract solution (5.0 mg/ml). Changes in catechin concentrations in saliva are shown in Table 2. Although none of the catechins

Table 2
Catechin concentrations (μg/ml) in siliva after mouthrinsing with green tea extract

	Before	Time after mouthrinsing (min)			
		1	10	30	60
GC	ND	26.69±5.67	9.58±2.49	6.41±1.78	5.39±1.36
EGC	ND	38.21±8.37	17.64±4.85	10.19±3.31	7.05±1.78
C	ND	11.92±2.69	3.41±0.90	1.75±0.49	1.65±0.41
EC	ND	20.50±4.36	7.42±1.93	4.34±1.33	2.80±0.76
EGCG	ND	165.14±36.52	52.13±15.20	24.72±7.92	16.06±4.49
GCG	ND	75.41±17.16	26.38±6.97	14.57±4.66	10.45±2.85
ECG	ND	43.43±9.69	15.41±3.99	7.57±2.48	5.24±1.53
CG	ND	11.78±2.58	4.79±1.23	2.61±0.89	1.91±0.57

Salivary catechins were determined after mouthrinsing with green tea extract (5.0 mg/ml). The value indicates mean±S.E. ($n=5$). ND=not detected.

were detected in saliva before mouthrinsing, significant amounts of eight catechins were retained in the oral cavity after mouthrinsing with the green tea extract. All the catechins were found to be present in saliva at μg/ml levels, even after 60 min. The quantitative analysis of the used green tea extract dissolved in 0.01 *M* HCl (0.1 mg/ml) revealed the catechin content in green tea (μg/mg) as follows: GC (75.90), EGC (78.97), C (25.50), EC (46.34), EGCG (273.37), GCG (117.73), ECG (68.34) and CG (17.55). Compared to the original catechin concentrations used for mouthrinsing (green tea extracts of identical catechin concentrations were used), the mean relative retentions of the eight catechins in saliva ($n=5$) ranged from 6.94 to 13.49, 2.95 to 6.22, 1.60 to 3.58 and 1.13 to 2.05% after 1, 10, 30 and 60 min, respectively.

The anticariogenic effect of catechins has been proposed to be based on their antibacterial activity against cariogenic bacteria [8], and on the inhibition of bacterial glucan synthesis and bacterial adherence to the tooth surface [9,10]. The minimum inhibitory concentrations of catechins to cariogenic *Streptococci* and the related Gram-positive bacteria are 250–500 μg/ml or less [8,24]. Among catechins, EGCG, ECG and GCG more intensively inhibit glucan synthesis by streptococcal glucosyltransferase [9,10]. EGCG and ECG, the major components in green tea extract, show an enzyme-inhibitory effect at 25–30 μg/ml or less [9]. They also inhibit the adherence of streptococcal cells to teeth at 50 μg/ml or less [9]. The present results suggest that these effective concentrations may be maintained in human saliva for at least up to 60 min after mouthrinsing with at least 5.0 mg/ml of green tea extract.

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