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Journal of Chromatography B, 692 (1997) 311–317

JOURNAL OF CHROMATOGRAPHY B

Application of a new high-performance liquid chromatographic method for measuring selected polyphenols in human plasma

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Received 29 May 1996; revised 24 October 1996; accepted 22 November 1996

Abstract

We developed a method to measure plasma levels of selected polyphenols before and after ingestion of green tea. Blood samples were obtained from four healthy women before and 30 and 50 min after the ingestion of 300 ml of green tea infusion. A 1-ml volume of plasma was hydrolysed with 0.5 *M* HCl–methanol (1:1, v/v) for 30 min at room temperature, extracted with ethyl acetate and separated by reversed-phase chromatography. Polyphenols were identified on the basis of their retention times and by spectrum analysis. Green tea caffeine has the same retention times as caffeic acid. Consumption of green tea produces a notable increase in the plasma levels of caffeine plus caffeic acid and the appearance of measurable levels of epigallocatechingallate. In conclusion, the method was found to have the requisite features of specificity and sensitivity for monitoring plasma levels of selected tea polyphenols.

Keywords: Polyphenols; Epigallocatechin; Catechin; Epigallocatechin gallate; Epicatechin; Caffeine; Epicatechingallate; Caffeic acid

1. Introduction

Polyphenols are important constituents of the human diet and are commonly found in vegetables (legumes, cereals, fruit) and in their by-products (tea, cider, oil, wine). There is ample evidence to suggest that polyphenols might play a role in the prevention of chronic diseases [1]. The protective mechanism is thought to be mainly associated with their antioxidant properties: superoxide anion scavenging, [2], peroxyl radical [3], hydroxyl radical and chelating catalyst metal ions [4].

Our knowledge about polyphenol bioavailability in man and its metabolic fate after absorption is scarce and conflicting. This dearth of information is partially due to the lack of sensitive and suitable methods for measuring polyphenols in plasma and body fluids, compared to the vast array of methods developed for their measurement in food [5].

This study proposes a specific new, highly sensitive and reliable method for measuring the plasma levels of catechin (CA), epigallocatechin (EGC), epigallocatechin gallate (EGCG), epicatechin (EC) and epicatechin gallate (ECG). These polyphenols are present in large amounts in tea (more than 35% by dry weight of tea leaves), but their nature depends on the manufacturing process that is used [6]. Green tea is rich in flavanols, mainly catechins, among which the following distribution was found: (+)-catechin (1.4%), (−)-epicatechin (5.8%), (−)-epigallocatechin (17.6%), (−)-epicatechingallate

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PII S0378-4347(97)00009-1

(12.5%), (−)-epigallocatechin gallate (53.9%) [7] and others (9.8%) [8].

To test the method, we measured the plasma levels of the selected polyphenols after green tea ingestion by four healthy women.

2. Experimental

2.1. Chemicals and solvents

Catechin, epigallocatechin, epicatechin gallate, epigallocatechin gallate and epicatechin were obtained from Extrasynthese (Genay, France) caffeine and caffeic acid were purchased from Sigma (St. Louis, MO, USA). All solvents were of analytical or HPLC grade and were obtained from Carlo Erba (Milan, Italy). Water was purified (18 MΩ) using a Milli-Q water purification system (Millipore, Milan, Italy).

2.2. Standard curves

The stock solutions containing 250 μg/ml of CA, EC, EGC, ECG and EGCG, 600 μg/ml caffeic acid and 100 μg/ml caffeine were obtained by dissolving pure standards in methanol and these were stored at −20°C until analysis. Standard solutions were obtained by diluting the stock solutions, at ratios of 1:10, 1:20, 1:40, 1:80 and 1:160, in methanol–water at pH 2.8 with ortho-phosphoric acid (3:2, v/v) for each phenolic compound.

2.3. Hydrolysis of sample

The hydrolysis step is crucial as the polyphenols present are in a conjugated form [9,10]. We tested the hydrolysis procedure using different HCl concentrations (2, 1 and 0.5 *M*), different temperatures (room temperature, 37°C) and different times (0, 10, 30 and 60 min). We observed that at high HCl concentrations, catechins and their gallates are degraded (20–80%). For example, the recovery (%) for EGCG at different HCl concentrations, at room temperature for 30 min is:

Time (min)	HCl concentration (*M*)	Recovery (%)	
		Room temperature	37°C
30	0.5	90.0	62
30	1.0	61.0	52
30	2.0	61.0	60

The optimum conditions for hydrolysis are reported below (Section 2.4).

2.4. Sample preparation

Plasma samples, obtained from four fasting subjects (see later), were submitted to extraction procedures as follows: a 1-ml volume of plasma was added to 1 ml of 0.5 *M* HCl–methanol (1:1, v/v), vortex-mixed for 30 s and was incubated for 30 min at room temperature. The mixture was extracted with 2 ml of ethyl acetate, the content of the tube was vortex-mixed for 3 min and centrifuged at 754 *g* for 5 min. The extraction procedure was repeated once more, the two organic layers were combined and then evaporated to dryness under a flow of nitrogen. The residue was re-dissolved in 250 μl of methanol–water at pH 2.8 with ortho-phosphoric acid (3:2, v/v).

2.5. HPLC analysis

A 20-μl volume of the extract and of each standard solution was injected into an autosampler (Perkin-Elmer ISS 200) of an HPLC system (Perkin-Elmer SEC-4). The polyphenols were separated on a Lichrospher 100 RP18 5 μm (25 cm×4.6 mm) column from Merck (Darmstadt, Germany) with a Perisorb Supelguard LC18 2 cm guard column from Supelco. The mobile phase consisted of two solvents: solvent A (demineralized water adjusted to pH 2.8 with phosphoric acid) and solvent B (methanol). We used the following gradient elution programme:

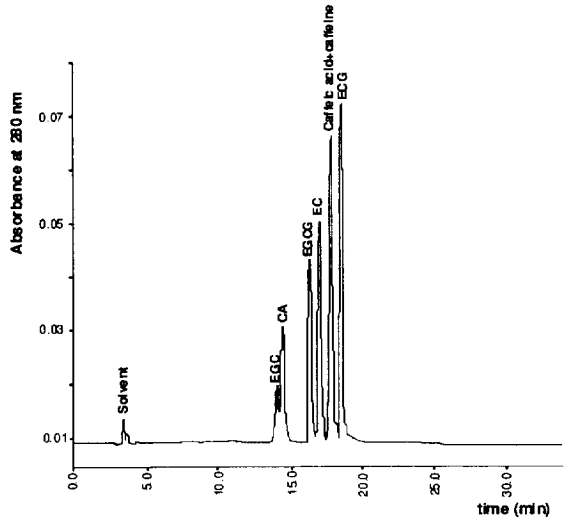

Fig. 1. Typical chromatographic pattern of a mixture of standards. Peak identification: EGC=epigallocatechin; CA=catechin; EGCG=epigallocatechin gallate; EC=epicatechin; caffeic acid+caffeine; ECG=epicatechin gallate.

Time (min)	Flow-rate (ml/min)	%B
1.0	1.0	13
11.0	1.0	60
14.0	0.8	60
34.0	0.8	80

Peaks were monitored with a spectrophotometric detector (Perkin-Elmer L.C. 95) set at 280 nm (AUFS 0.001, time response 2000), and were recorded and analysed with a personal computer (PE Nelson 1020).

Peak identification was further confirmed using a diode array detector (Perkin-Elmer LC 235) and an electrochemical coularray detector with potentials made up of a symmetric array from 60 to 840 mV (ESA Chelmsford, USA).

The entire procedure took approximately 50 min to complete (including the stabilisation time).

2.6. Calculations

Fig. 1 shows the typical chromatographic pattern of the standard mixture. The retention times of the samples were compared to those of a standard mixture for identification. Quantification of a single compound was performed by the external standard method and the results are expressed as μg/ml.

2.7. Linearity of HPLC system and its sensitivity

Linear regression was obtained by plotting the peak-height ratio of a series of dilutions for each phenolic compound against the known concentration of the analyte in the stock standard solution. The regression lines, expressed as correlation coefficients, were linear and close to one (r=0.999) for each parameter in the studied range, as shown in Table 1.

Sensitivity (defined as the lowest measurable concentration of a compound in the sample), was estimated as the concentration that generated a peak with a height that was at least three-times higher than the baseline noise range (Table 1).

2.8. Recovery and reproducibility

The percentage recovery of the method was established by measuring the percentage recovery after the addition of known amounts of standard to a reagent blank and to a pool of human plasma (Table

Table 1
Linearity and sensitivity of the detection method for selected phenolic compounds

Compound	Range studied (μg/ml)	Correlation coefficient (r)	Sensitivity (μg/ml)
Epigallocatechin	1.0–80.0	0.997	1.0
Catechin	0.5–18.0	0.980	0.5
Epigallocatechin gallate	0.9–25.0	0.999	0.9
Epicatechin	1.1–8.8	0.999	1.1
Caffeine plus caffeic acid	0.7–60.0	0.998	0.7
Epicatechingallate	1.3–20.0	0.999	1.3

Table 2
Recovery (%) of added standards from reagent blank and plasma

Compound	Reagent blank[b]		Analytical recovery from plasma[b]	
	No.[a]	%	No.[a]	%
Epigallocatechin	8	97.2±3.3	8	96.0±6.0
Catechin	8	96.9±11.2	8	93.5±15.6
Epigallocatechin gallate	9	99.2±7.5	6	100.0±5.9
Epicatechin	12	93.5±8.2	6	98.7±8.8
Caffeine plus caffeic acid	8	102.4±3.3	6	97.1±7.8
Epicatechin gallate	10	95.3±5.7	9	101.1±7.1

[a] No.=number of runs.
[b] Mean of triplicate assays.

2). The values obtained from the reagent blank indicate that each compound is quantitatively extracted in ethyl acetate. Each determination was the result of duplicate or triplicate analyses conducted on different days.

Reproducibility was measured by the triplicate analysis of human plasma on the same day (intra-day C.V.). Deep frozen plasma was measured for ten days to test the day-to-day reproducibility of the method (inter-day C.V.) (Table 3). The intra- and inter-day coefficients of variation were less than 10% in all cases, except for EGC and CA.

2.9. Applicability to human studies

The suitability of the method for measuring selected phenols in human plasma was tested on fasting volunteers ingesting green tea. Analysis of the flavonol profile of the green tea drink by our HPLC method is shown in Fig. 2. Each cup of green tea contains EGC (90±7.6 mg), CA (30±1.3 mg);

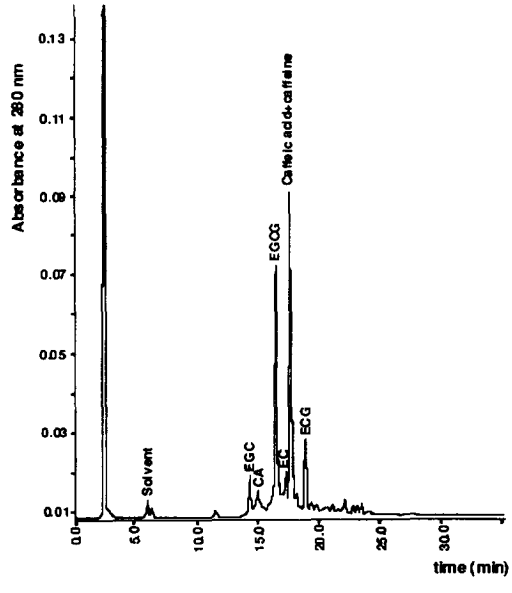

Fig. 2. Typical chromatogram of the extracted polyphenols from diluted green tea.

Table 3
Reproducibility [mean±S.D. and C.V. (%)] of added standards (μg/ml) to pooled plasma

Compound	Intra-day[b]			Inter-day[c]		
	No.[a]	X	C.V. (%)	No.[a]	X	C.V. (%)
Epigallocatechin	3	4.4±0.3	7.9	8	4.4±0.6	14.6
Catechin	3	3.7±0.4	9.4	8	3.8±0.6	13.5
Epigallocatechin gallate	3	6.8±0.2	3.0	6	6.9±0.4	6.2
Epicatechin	3	4.4±0.2	3.6	6	4.5±0.2	5.0
Caffeine plus caffeic acid	3	3.3±0.1	2.1	6	3.3±0.3	8.3
Epicatechin gallate	3	9.3±0.6	6.0	9	9.9±0.9	9.0

[a] No.=number of runs.
[b] Mean of triplicate assays.
[c] Single or duplicate assay.

Table 4
Physical characteristics of the subjects on entry into the study

Subject	Age (years)	Weight (kg)	Height (cm)	BMI[a] (kg/m^2)
A	32	58.0	163	21.8
B	29	56.0	168	19.8
C	32	52.0	163	19.5
D	26	47.0	160	18.4
Mean	29.8±2.9	53.3±4.9	164±0.04	19.5±1.4

[a] BMI: body mass index.

EGCG (118.0±12.6 mg); EC (29.5±0.44 mg); caffeine+caffeic acid (110.8±4.2 mg) and ECG (30.6±0.7 mg). These values are the mean of four analyses.

Four healthy adult women were recruited from the personnel of the National Institute of Nutrition in Rome. These volunteers stopped drinking tea three days before the experiment but they did take coffee and consumed a regular diet. The individual and mean values of age (years), weight (kg), height (cm), and body fatness, expressed as body mass index (BMI: kg/m^2), of subjects participating in the study are shown in Table 4.

Venous blood (5 ml) was collected at 8 a.m. (baseline) in vacutainers containing EDTA, after an overnight fast. Subsequently, all the subjects received a cup of green tea (300 ml) prepared by infusing 6 g of dry tea leaves (Birko Chinese green tea, KI) in tap water at 100°C for 3 min. Venous blood was obtained 30 and 50 min after tea drinking. One subject underwent further blood sampling 120 min after ingestion of the tea. Blood samples were centrifuged for 15 min at 644 *g* and the plasma was stored at −40°C prior to analysis. During the test, subjects did not ingest other beverages or food, with the exception of water.

3. Results

Fig. 3 shows a comparison of plasma polyphenol levels before and after the ingestion of green tea. The analysis of plasma in the fasting state revealed the presence of caffeine+caffeic acid, which co-eluted (Fig. 3). After green tea ingestion, a new peak was detected in plasma and was identified as EGCG.

Identification of these peaks was further confirmed

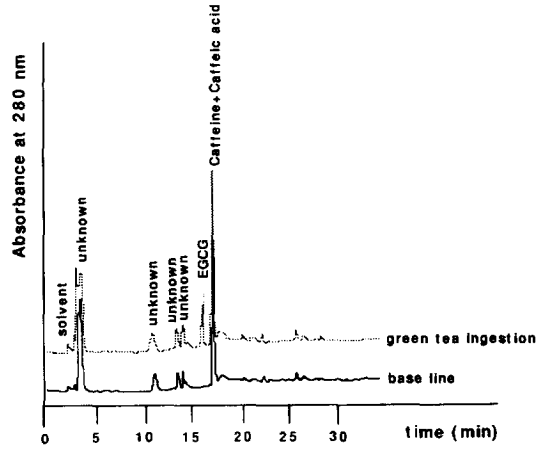

Fig. 3. Comparison of the plasma levels of polyphenols before and after ingestion of green tea.

by a UV spectroscopy with diode array detection. The UV spectrum of EGCG in plasma after green tea ingestion (A) was compared with the spectrum of the pure EGCG standard (B). Spectra A and B have maxima at 275 nm; Fig. 4 shows their similarities. We repeated the analysis using the coularray electrochemical detector, which confirmed the first result. We conducted the same UV spectra analysis for caffeine and observed that plasma had a maxima at 271 nm, which was the same as the caffeine standard (data not shown).

Figs. 5 and 6 show the changes in plasma levels of EGCG and caffeine+caffeic acid following the

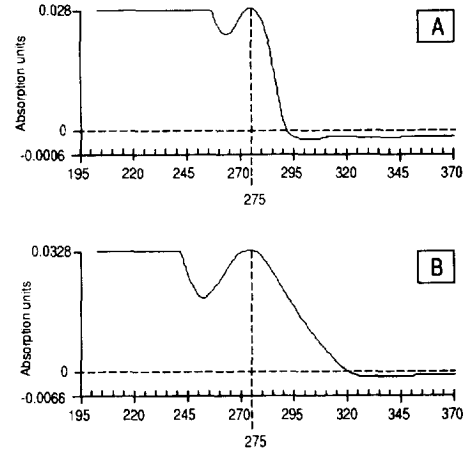

Fig. 4. Spectral analysis of the EGCG peak during a HPLC run, evaluated in plasma and a standard solution.

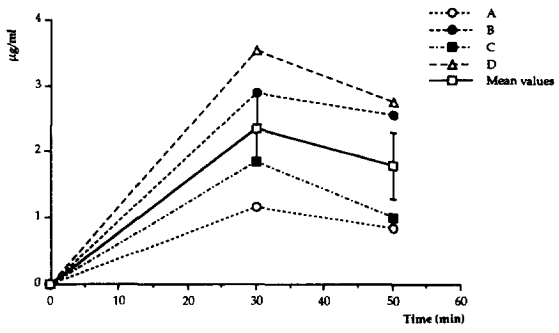

Fig. 5. Plasma concentration of EGCG before and after green tea ingestion at timed intervals. Each line corresponds to one subject. The solid line is the mean±S.E.M. of all subjects.

ingestion of green tea. The results are expressed as μg/ml and each line corresponds to one subject while the solid line represents the mean±S.E.M. for all subjects. EGCG (Fig. 5), which was absent in plasma from fasting individuals, was detected at 30 min (mean value of 2.36±0.52), and decreased at 50 min (mean value of 1.79±0.50). There is a marked inter-individual variability with a two-fold difference in the peak values between the lowest and the highest response. The caffeine+caffeic acid present in plasma from fasting individuals (Fig. 6) almost doubled at 30 min (from 16.2±10.8 to 44.5±16.5) after tea ingestion and continued to increase, although at a slower rate, up to 50 min (51.3±17.2) after ingestion. Also, for caffeine+caffeic acid there was marked inter-individual variability, although most of this was caused by the high baseline values

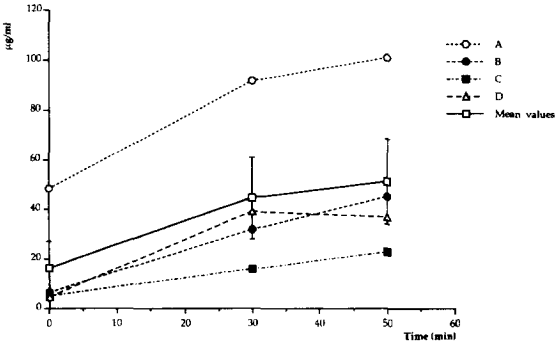

Fig. 6. Plasma concentration of caffeine+caffeic acid before and after green tea ingestion at timed intervals. Each line corresponds to one subject. The solid line is the mean±S.E.M. of all subjects.

of one subject, a regular consumer of large quantities of coffee (>five cups per day).

The measurement of plasma polyphenols 120 min after tea ingestion revealed that the presence of EGCG rapidly disappeared, as it had fallen to undetectable levels, while caffeine plus caffeic acid had decreased appreciably, without returning to baseline values (data not shown).

4. Discussion and conclusion

The possible role of dietary polyphenols in modulating oxidative stress in man is attracting wide interest. However, the mechanism of flavonoid absorption and transport is still unclear, and data on polyphenol plasma levels are scarce and conflicting, due to the lack of accurate and reliable methods for their measurement in body fluids.

In this study, we developed a method for evaluating selected tea polyphenols in human plasma. An advantage of the proposed reversed-phase HPLC method is that it can be performed easily in all laboratories. The method is highly effective, yielding reproducible and reliable results, is rapid, thus making it suitable for studies conducted on large groups. The handling of samples is easy, simple and can be performed automatically. The procedure evaluates the free and conjugated forms of polyphenol, although it is probably not specific for the sulphate and glucuronate forms. These forms originate in the liver, which has the capacity to oxidize, reduce, methylate or conjugate (sulphate and glucuronate) polyphenols [11].

Analysis of human plasma after the ingestion of green tea provided additional information on the kinetics of polyphenol absorption. In our study, it appears that green tea polyphenols are quickly absorbed and can be detected in plasma. The appearance or rise of EGCG and caffeine+caffeic acid in plasma confirms indirect evidence that they are rapidly absorbed by humans [12]. However, we found that there are large inter-individual differences (ranging from 22 to 66%), despite the standard conditions under which the test was conducted, suggesting that the absorption process might be modulated by intrinsic biological factors (delay in gastric emptying, gastric activity, etc.).

Our results are in agreement with a previous observation that EGCG appears in plasma 30 min after oral administration of EGCG to rats [13]. Recently, Lee et al. [14] detected EGCG, EGC and EC in human plasma 1 h after the ingestion of 1.2 g of decaffeinated green tea (in 200 ml of water), using a novel HPLC method.

In conclusion, a highly precise, sensitive, reproducible and reliable HPLC technique has been developed to study bioavailability and the levels of selected polyphenols in biological fluids.

Acknowledgments

This work was supported by the National Research Council of Italy, Special Project RAISA, Sub project 4.

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