



Simultaneous determination of the major active components of tea polyphenols in rat plasma by a simple and specific HPLC assay

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ABSTRACT

A simple and specific HPLC assay for simultaneous determination of two major active components (–) epigallocatechin-3-gallate (EGCG), and (–) epicatechin-3-gallate (ECG) of tea polyphenols (TP) in rat plasma was developed and validated. Following addition of resorcinol as internal standard (IS) the analytes were isolated from rat plasma by liquid–liquid extraction with ethyl acetate. The chromatographic separation was achieved on a reversed-phase C18 column using an isocratic mobile phase consisting of 0.1% citric acid + CH₃CN (86:14, v/v) running at flow rate of 1.5 mL/min. The effluent was monitored at a wavelength of 280 nm. EGCG, ECG and IS were well separated from each other and free from interference from blank plasma and other components in TP as well as metabolites post-dosing. The calibration curve was constructed by plotting peak area ratio of analytes to IS vs. concentration. The method showed good linearity over range of 0.5–300 µg/mL for EGCG and 0.1–60 µg/mL for ECG ($r > 0.999$). The intra- and inter-day precision (R.S.D.) was better than 6 and 12%, respectively. Assay accuracy was better than 94.78% for both compounds. Extraction recovery at QC samples was between 85.73 and 91.93% for EGCG and 79.08 and 86.51% for ECG. The developed method was successfully used to simultaneously measure plasma concentrations of EGCG and ECG after intravenous administration of TP to rats and yielded two typical biexponential decay concentration–time curves.

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1. Introduction

Tea polyphenols (TP) is a group of polyphenolic compounds extracted from green tea (*Camellia sinensis*), a widely consumed beverage in many countries around world, especially in China and Japan. It has been reported that the main active components of TP are flavanols, commonly known as tea catechins including (–) epigallocatechin-3-gallate (EGCG), (–) epicatechin-3-gallate (ECG), (–) epigallocatechin (EGC) and (–) epicatechin (EC) (Fig. 1), EGCG being the most active and abundant, ECG next, EC the least [1,2].

Over the past two decades, numerous studies have revealed that TP is the potent free radical scavenger and has wide health-promoting properties, such as anti-atherosclerosis, anti-obesity, anti-diabetic, anti-aging and anti-irradiation effects [3–7]; especially, the cancer chemopreventive effects and anti-AIDS effects of TP have attracted ever-increasing attention [8–10].

We have been studying TP recently, and our findings indicated that TP are much more potent than vitamin C, an established water-soluble antioxidant, in anti-free radical activity, and are powerful in protecting multiple organs, in particular brain from injuries induced by ischemia and reperfusion [11–14], showing a good potential of clinical application.

In view of these findings, we aimed to develop TP as a novel cerebro-protective agent given by intravenous route for the treatment of cerebral apoplexy as well as its sequelae; for this end, both pharmacodynamic and pharmacokinetic studies are essential. To date, a few papers have reported on HPLC analysis of TP in biological fluids and tissues; for instance, HPLC-ECD method has been developed for determination of catechins in human urine subsequent to tea ingestion [15], HPLC-UV for simultaneous analysis of catechins and other constituents in green tea using an internal standard and a gradient mobile phase and two columns in series [16], HPLC-CEAD for determination of catechins and catechin gallates in biological fluids and in tissues [17,18], LC-MS for analysis catechins and their metabolites [19]. However, these detections are expensive and not usually available in common laboratories. Dr. Chen et al. [20] have developed an HPLC method for simultaneous determination of EGCG, ECG and EC in rat plasma; nevertheless, this method took advantage of a

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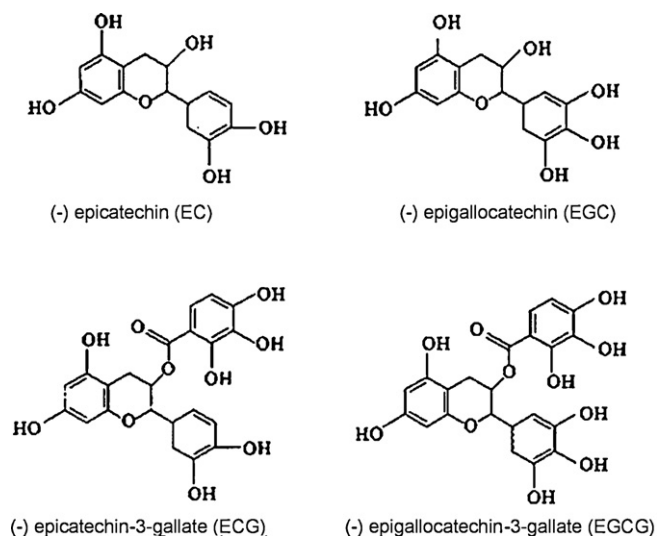


Fig. 1. Chemical structures of EC, EGC, ECG and EGCG.

complicated linear ternary gradient mobile phase and enzymatic hydrolysis procedure, and measured the total amount of conjugated and non-conjugated forms of catechins in plasma, based on external standard quantification method. In general, only free forms of drug are thought to be pharmacologically active, so, determination of free non-conjugated forms of catechins might be more advisable. So far, no report has been found concerning HPLC determination of active catechins of TP with isocratic elution mode. Considering the above, we have developed a simple and specific isocratic reversed-phase HPLC-UV assay for the simultaneous determination of the two major active parent (non-conjugated forms) components, i.e. EGCG and ECG, of TP in plasma of rats. This assay takes advantage of liquid–liquid extraction technique for pre-treatment of plasma samples and internal standard method for quantification of free form of each of EGCG and ECG, and has been validated to be useful in the pharmacokinetic study of the EGCG and ECG in plasma of rats receiving intravenous administration of TP.

2. Experimental

2.1. Chemicals and reagents

TP, as an extract from green tea, was purchased from Wu Yuan Tea Plantation (Jiang Xi Province, China), which was obtained by extraction with ethanol, chloroform and ethyl acetate as organic solvents and followed by purification with silica gel column chromatography; its purity was >98%, determined based on spectrophotometry with ferrous tartrate as chromogenic agent; and HPLC assay in our laboratory showed the TP contained 63.84% (g/g) EGCG and 7.30% ECG. EGCG and ECG reference substances were purchased from Shanghai U-sea Biotech Co. Ltd. (Shanghai, China) and have purity of 97.0 and 98.0%, respectively, checked with the standards provided by Sigma (St. Louis, MO, USA). Resorcinol used as internal standard was a gift from Department of Chemical Pharmacy, Dalian University of Technology, its purity was tested and found to be better than 99.0% by HPLC. Acetonitrile (CH₃CN), methanol (MeOH), all of HPLC grade, were purchased from Tedia Company (USA). Ethyl acetate (EtOAc) and all other chemicals used were of analytical grade. The double-distilled water was used for preparing mobile phase.

2.2. Chromatographic conditions

The liquid chromatographic system included an Agilent 1100 HPLC chromatograph equipped with a quaternary pump, an online degasser, a UV-detector and a refrigerated autosampler. System control and data processing were carried out by a ChemStation 32 Software. The analytes were eluted on a Chromasil C18 analytical column (250 mm × 4.6 mm ID, 5 μm) (Elite, Dalian, China) protected by a Chromasil C18 precolumn (20 mm × 4.6 mm ID, 10 μm) (Elite, Dalian, China) maintained at room temperature using an isocratic mobile phase composed of 0.1% citric acid + CH₃CN (86:14, v/v) running at a flow rate of 1.5 mL/min. The effluent was monitored at a wavelength of 280 nm.

2.3. Preparation of standards

A mixture stock solutions of EGCG standard (12 mg/mL) and ECG standard (2.4 mg/mL) were prepared in MeOH. The internal standard solution containing 75 μg/mL of resorcinol was prepared in the same solvent. These solutions were stored at −20 °C. The working standard solutions containing 6000, 3500, 2000, 1000, 400, 20, 10 μg of EGCG and 1200, 700, 400, 200, 80, 4, 2 μg of ECG per mL, respectively, were freshly prepared by serially diluting the above mixture solution of standards with double-distilled water.

2.4. Experimental animals

Male Sprague–Dawley rats weighing of 200–250 g were obtained from the Animal Center of Dalian Medical University, and were housed at 25 °C with a 12-h light and dark cycle with free access to a commercial rat chow and water. The experiments started after acclimation for at least 1 week. TP solution was made freshly in saline and was intravenously administered to rats (*n* = 6) via tail vein at a bolus dose of 100 mg/kg, equivalent to 63.84 and 7.30 mg/kg of EGCG and ECG, respectively. Aliquots of blood samples were collected from orbital sinus by venipuncture pre-dose and 2, 5, 10, 20, 40, 60, 90, 120, 240 and 360 min post-dose and centrifuged immediately at 4 °C at 3000 × *g* for 10 min. The resultant plasma samples were stored at −20 °C until analysis.

2.5. Plasma sample preparation

A 200 μL of rat plasma sample was transferred into an Eppendorf tube containing 10 μL each of IS and 20% vitamin C solution. The resultant mixture was extracted twice with 400 μL of EtOAc each by vortex-mixing for 0.5 min and centrifuged at 3000 × *g* for 10 min at 4 °C. The upper organic phase was transferred into another Eppendorf tube and evaporated to dryness under a gentle N₂ stream at 50 °C in a water bath. The residue was reconstituted in 100 μL of a 10% CH₃CN aqueous solution. Following centrifuging at 3000 × *g* for 10 min at 4 °C, 60 μL of supernatant was injected onto chromatograph for analysis.

2.6. Plasma calibration curves

Seven portions of 200 μL of blank rat plasma were spiked with 10 μL each of appropriate working standard solutions to yield plasma standard samples containing 300, 175, 100, 50, 20, 1.0, 0.5 μg EGCG per mL plus 60, 35, 20, 10, 4.0, 0.2, 0.1 μg ECG per mL, respectively, and then treated as described under Section 2.5 above. The calibration curves were constructed by plotting the peak area ratios of each analyte/IS vs. analyte concentration in plasma. The regression equation of the calibration curve of each analyte was calculated by weighed least square regression method with 1/*C*² as weighing factor.

2.7. Quantification of EGCG and ECG in rat plasma samples

The frozen rat plasma samples were thawed naturally at room temperature and then treated as described under Section 2.5. Then, peak area ratio of analyte/IS was calculated from recorded chromatogram. The concentration of each analyte could be read from the accompanying calibration curve.

2.8. Validation of methodology

This HPLC assay was validated according to the Guiding Principle issued by China SFDA [21], using quality control (QC) samples containing EGCG + ECG, which were prepared as described under Section 2.6, at high ($175 + 35 \mu\text{g/mL}$), middle ($50 + 10 \mu\text{g/mL}$) and low ($1.0 + 0.2 \mu\text{g/mL}$) concentrations for 5 repeated analysis, separately.

The extraction recovery of each analyte and IS was assessed by direct comparison of respective peak area obtained from extracted QC sample vs. from unextracted plasma-free standard solution containing the concentration identical to QC sample. Intra-day and inter-day precisions were determined by calculating the relative standard deviation (R.S.D.) for 5 determinations at each concentration of 3 QC samples on a single assay day and on 5 separate assay days. The assay accuracy was assessed by calculating the estimated concentrations, based on the regression equation of the calibration curve, as a percent of the nominal concentrations.

The stability of plasma samples was investigated at storage conditions of -20°C and room temperature using QC samples, and assessed by calculating the concentration measured at different time as a percent of the concentration measured at time 0.

The spiked each analyte concentration in blank plasma that gave a signal to noise ratio of 3:1 was considered the limit of detection (LOD).

2.9. Pharmacokinetic analysis

The pharmacokinetic parameters of EGCG and ECG were calculated by 3P97 Program developed by China Mathematical Pharmacological Society.

3. Results and discussion

3.1. Method development

An appropriate internal standard is needed for accurate quantification of analytes. In this study, the selection of the internal standard was an arduous and hard process. We tested nevadensin, gastrodin, quercetin, psoralen, ethyl hydroxybenzoate, protocatechualdehyde and phloroglucinol, but all failed, because of the unsuitable retention time or interference from the endogenous matrix. Resorcinol was finally selected as the internal standard because it had a suitable retention time and a good resolution from EGCG and ECG under the chromatographic conditions in this study.

Various extraction solvents were evaluated to acquire the best analytical results. EtoAc was found to provide the highest recoveries compared with other tested solvents such as diethyl ether, chloroform and anhydrous ethanol. Besides, two-extraction with EtoAc was found much better than once-extraction. Pre-treatment of plasma samples with trichloroacetic acid for deproteinization was associated with very low recoveries.

The effect of composition of mobile phase on retention and resolution of EGCG and ECG were investigated. After optimization, the mobile phase consisting of 0.1% citric acid + CH_3CN (86:14, v/v) was chosen as it gave a shortest retention time, better

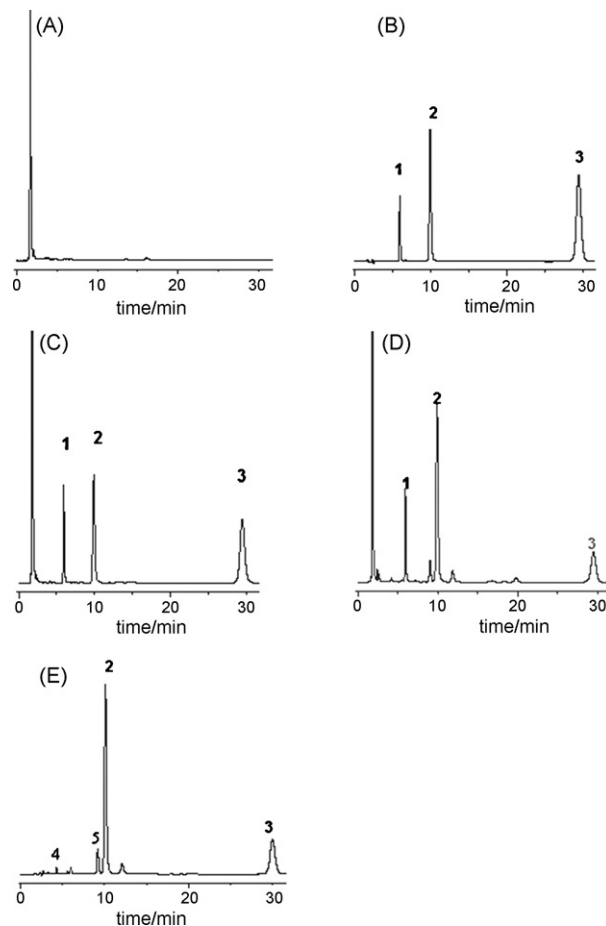


Fig. 2. Chromatograms—(A) Blank plasma; (B) EGCG, ECG and internal standard (resorcinol); (C) blank plasma spiked with EGCG, ECG and internal standard (resorcinol); (D) plasma sample from a rat after intravenous administration of 100 mg/kg dose of TP; (E) TP pharmaceutical product: (1) internal standard (t_R 6.0 min); (2) EGCG (t_R 9.9 min); (3) ECG (t_R 29.5 min); (4) EGC (t_R 4.8 min); (5) EC (t_R 9.4 min).

shapes of peaks, and good resolution between EGCG, ECG, IS and matrix.

3.2. Specificity

From Fig. 2 it could be seen that the EGCG, ECG and IS were separated well from each other with retention time (t_R) of 9.9, 29.5 and 6.0 min, respectively, and resolution (R_s) of 3.6 between EGCG and IS and 11.8 between EGCG and ECG. There was no interference from blank plasma and from metabolites. What is more, it was also found from Fig. 2E that the other components or impurities of TP raw material did not interfere with analytes and IS; the peaks with t_R 4.8 and 9.4 min were shown to be identical to standards of EGC and EC.

3.3. Linearity and limit of quantitation

The plasma calibration curve of each analyte was found to be linear over studied range of $0.5\text{--}300 \mu\text{g/mL}$ for EGCG and $0.1\text{--}60 \mu\text{g/mL}$ for ECG, with weighed linear regression equation of Y (peak area ratio of analyte/IS) = $0.05418 + 0.05254X$ (concentration, $\mu\text{g/mL}$) for EGCG ($r = 0.9994$, $n = 5$) and $Y = 0.01183 + 0.2354X$ ($r = 0.9993$, $n = 5$) for ECG. The limit of detection (LOD) was 0.125 and $0.025 \mu\text{g/mL}$ ($S/N = 3:1$), and the limit of quantitation (LOQ) was 0.5 and $0.1 \mu\text{g/mL}$, respectively. During course of construction

Table 1

Precision, accuracy and recovery of EGCG and ECG in rat plasma.

Analyte	Added ($\mu\text{g/mL}$)	Intra-day		Inter-day		Extraction recovery (%)	Accuracy (%)
		Found	R.S.D. (%)	Found	R.S.D. (%)		
EGCG	175	184.84 \pm 2.20	1.31	180.18 \pm 7.98	4.43	85.73 \pm 2.55	105.62 \pm 1.38
	50	47.39 \pm 2.09	4.41	43.85 \pm 3.22	7.34	86.90 \pm 0.21	94.78 \pm 4.18
	1	1.09 \pm 0.05	4.59	1.07 \pm 0.12	11.21	91.93 \pm 2.14	109.00 \pm 5.01
ECG	35	38.72 \pm 0.62	1.60	36.18 \pm 3.14	8.68	83.67 \pm 2.26	110.63 \pm 1.77
	10	10.12 \pm 0.58	5.73	9.96 \pm 1.04	10.44	79.08 \pm 1.86	101.20 \pm 5.82
	0.2	0.19 \pm 0.01	5.26	0.18 \pm 0.02	11.11	86.51 \pm 3.37	95.00 \pm 5.03

of calibration curve, the following four procedures were tried: (1) usual least square linear regression, (2) weighed least square linear regression with weighing factor of $1/C$, (3) weighed least square linear regression with weighing factor of $1/C^2$ and (4) separate two range linear regression. The third procedure was found much superior to others and was therefore chosen in order to avoid undue bias to the low concentrations of the calibration curve by the high concentration.

3.4. Precision, accuracy and recovery

Table 1 shows that the intra- and inter-day precision (R.S.D.) of EGCG and ECG in plasma samples were better than 6 and 12%, respectively; assay accuracy was better than 94.78% for both compounds. Extraction recovery at QC samples was between 85.73 and 91.93% for EGCG and 79.08–86.51% for ECG. The extraction recovery of IS was found to be 93.68% with R.S.D. of 5.90% ($n = 15$).

3.5. Stability of plasma samples

It has been demonstrated that when plasma samples were stored at room temperature the relative content was measured to be 100, 97.35, 97.33, 97.92% for EGCG and 100, 97.22, 96.43, 93.70% for ECG at 0, 2, 4 and 8 h, respectively; when stored at -20°C , the relative content to be 100, 94.28, 101.67, 102.15 and 95.68% for EGCG and 100, 94.23, 99.75, 100.07 and 94.85% for ECG at d0, d2, d7, d14 and d21, respectively, indicating that the plasma samples were stable for at least 8 h and 21 days when stored at room temperature and -20°C , respectively.

3.6. Assay application

Fig. 3 presents mean plasma drug concentration–time curves of EGCG and ECG, showing that the two compounds disappeared from plasma in a biexponential manner and, thus, pharmacokinetically behaved as a two-compartment model with the first-order kinetics. The main pharmacokinetic parameters of EGCG and ECG were estimated as follows: $t_{1/2\beta}$ 68.6 and 21.4 min, V_d 2.21 and 0.99 L/kg, CL 0.022 and 0.034 L/(kg min), respectively, indicating that EGCG and ECG eliminated rapidly from body of rats after intravenous administration of TP.

In this assay, two major active catechins are measured, other catechins EC and EGC contained in TP raw material were not analyzed. This is because EC and EGC are low not only in activity but also in content (<5 and $<1\%$, respectively, as evidenced by chromatogram of TP in Fig. 2E) as compared with EGCG and ECG. However, this assay can chromatographically differentiate above four catechins, thus making it possible for us to observe if there is any potential metabolic conversion of the two analytes in rat plasma.

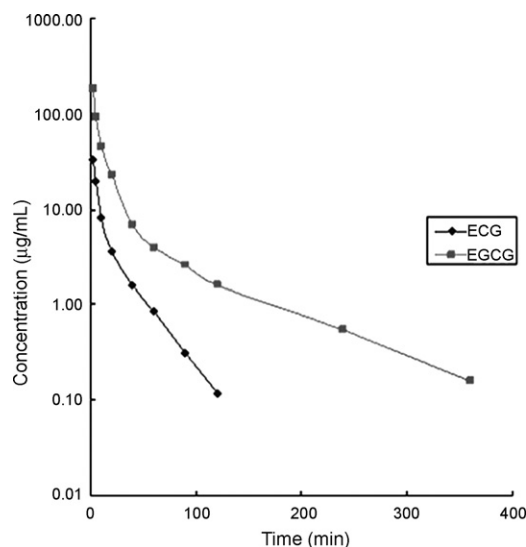


Fig. 3. Mean plasma concentration–time profiles derived after intravenous administration of 100 mg/kg of TP to rats ($n = 6$).

4. Conclusion

The present article describes an isocratic HPLC assay and internal standard quantification for simultaneous determination of two active ingredients EGCG and ECG in plasma of rats administered intravenously TP extracted from green tea, which was validated to be simple, specific, precise and thus completely suitable for pharmacokinetic study of TP.

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