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Liquid chromatography with multi-channel electrochemical detection for the determination of epigallocatechin gallate in rat plasma utilizing an automated blood sampling device

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Abstract

A liquid chromatography method with multi-channel electrochemical detection was developed for the determination of epigallocatechin gallate (EGCG) in rat plasma. After administration of EGCG, blood samples were periodically collected by Culex (an automated blood sampling robot). EGCG was extracted from 50 μl of diluted blood (blood and saline at a ratio of 1:1) with ethyl acetate. Chromatographic separation was achieved within 10 min using a C_8 (150×4.6 mm) 5 μm column with a mobile phase containing 20 m*M* sodium monochloroacetate, pH 2.8 and 12% acetonitrile at a flow-rate of 1.2 ml/min. A four-channel detector with glassy carbon electrodes was used with applied potentials of +700, 600, 500, 400 mV vs. Ag/AgCl. The limit of detection was 2 ng/ml at a signal-to-noise ratio of 3:1 and the limit of quantitation was 5 ng/ml. The calibration curve was linear over the range of 5–800 ng/ml. The intra- and inter-assay precisions were in the range of 1.3–4.5% and 2.2–4.4%, respectively. Using this method it was possible to determine plasma concentration following a single dose of EGCG to rats with good accuracy and precision. Thus the pharmacokinetic properties of EGCG in rats can be examined for intravenous, intraperitoneal and oral dosing.

Keywords: Epigallocatechin gallate

1. Introduction

Tea is a widely consumed beverage. Epidemiological studies have demonstrated an association between tea consumption and decreased cancer risk [1]. Polyphenols in tea are believed to be responsible for many of its health benefits. Among tea polyphenols, epigallocatechin gallate (EGCG) is the most abundant in amount and is assumed to be the major effective compound. Animal studies indicated that green tea polyphenols inhibit carcinogen-induced skin, lung, forestomach, esophagus, duodenum and colon tumors in rodents and inhibit TPA (12-O-tetradecanoylphorbol-13-acetate)-induced skin tumor promotion in mice [2–4]. EGCG shows a pronounced growth inhibitory effect on cancerous cells but not on their normal counterparts [5]. EGCG was reported to reduce cholesterol, glucose level and food intake in rat [6]. It is suggested that long term consumption of green tea may influence the inci-

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dence of obesity, diabetes and cardiovascular disease [6]. It was also reported that tea polyphenols are neuroprotective due to their antioxidative property [7,8].

Much attention has been paid to the studies of physiological effects of green tea polyphenols. In order to understand the mechanism of the potential anticancer effect of tea polyphenols, it is important to obtain information on their phamacokinetic properties. Several papers have reported pharmacokinetic studies of tea polyphenols in rat [9–11] and human [12–14]. Tsuchiya and co-workers have reported an LC method to determine the concentration of tea catechines in human saliva and plasma [15,16]. Among these papers, liquid chromatography (LC)–UV, LC–CL (chemiluminescence) and LC–electrochemical detection (EC) methods [17,18] have been used. As one of the major components in tea, caffeine can interfere with the UV analysis of catechins. LC–EC methods showed improved selectivity because the interference of caffeine was reduced due to its low electrochemical activity.

This paper describes a reversed-phase LC method with multi-channel electrochemical detection to determine EGCG in rat plasma. Compared to traditional electrochemical detection, multi-channel electrochemical detection provided a fast and easy way to both recognize and often eliminate interferences from endogenous compounds in plasma. This provided a selective and sensitive approach for the peak identity and determination of EGCG in plasma. This method has been applied to evaluate the pharmacokinetics of EGCG.

2. Experimental

2.1. Apparatus

The LC–EC system was comprised of a chromatographic pump (PM-92e, BAS, West Lafayette, IN, USA), an in-line filter (0.5 μm, Rheodyne) before the analytical column, a C_8 5 μm column (150×4.6 mm, YMC, Milford, MA, USA), a CMA/200 refrigerated autosampler (CMA, Stockholm, Sweden), a multi-channel amperometric detector (epsilon, BAS) using a square array of four 2 mm glassy carbon working electrodes and referenced to a Ag/AgCl electrode. The injection loop volume was 50 μl. Applied potentials of +700, 600, 500 and 400 mV vs. Ag/AgCl were utilized. Data was acquired and integrated using BAS ChromGraph version 2.0.01 chromatography software.

The rat blood collecting system consisted of a freely moving rat containment device (Raturn, BAS) [19], and an experimental automated blood sampler (Culex, BAS). The blood samples were collected into capped 300-μl vials in a fraction collector (HoneyComb, BAS) maintained at 4°C.

2.2. Chemicals and reagents

EGCG was purchased from Sigma (St. Louis, MO, USA). Acetonitrile and ethyl acetate were of HPLC grade (Burdick & Jackon, Muskegon, MI, USA). Reagent-grade water was prepared from laboratory-deionized water using a NANOpure system (Barnstead/Thermolyne, Dubuque, IA, USA). Chloroacetic acid (MCAA) of analytical grade was purchased from Aldrich (Milwaukee, WI, USA).

2.3. Standard curve and quality control samples

EGCG was dissolved in 10 m*M* HCl at a concentration of 1 mg/ml and stored in the dark at 4°C until used. EGCG was added to pooled rat plasma to yield final concentrations of 5, 10, 100, 400, 800 ng/ml. These spiked samples were used to construct the standard curve. Quality control (QC) samples were prepared in pooled rat plasma to contain concentrations of EGCG within the standard curve range.

2.4. Sample preparation

A total of 150 μl of blood solution, which contained 75 μl of rat blood and 75 μl of physiological saline was collected for each sample. The solution was centrifuged at 2000 *g* for 10 min, then two equal volumes of 50 μl supernatant solution were transferred to two 1.7-ml centrifuge tubes, one was analyzed, the other was frozen at −20°C as a backup sample. Ethyl acetate (600 μl) was added to the 50

μl of sample, vortex-mixed for 2 min, and centrifuged for 6 min at 5600 *g*. Following centrifugation, 500 μl of the clear supernatant was transferred to another centrifuge tube, dried under nitrogen, reconstituted with 50 μl of mobile phase. A volume of 20 μl of the solution was injected by autosampler.

2.5. Assay validation

2.5.1. Calibration

A calibration curve was constructed by plotting peak height (nA) of the analyte vs. the analyte's concentration (ng/ml). The weighted ($1/x$) linear regression was fitted over the concentration range 5–800 ng/ml.

2.5.2. Accuracy and precision

The inter- and intra-assay validation was performed by assaying QC samples (50, 200, and 400 ng/ml) with three replicates on 3 different days. The accuracy and precision were reported as the bias (%) and the RSD (%), respectively.

2.6. Preliminary animal study

Traditional pharmacokinetic studies involve intermittent blood sampling and subsequent determination of blood or plasma drug concentrations. Automated blood sampling as a continuous blood withdrawal approach provided a means of accurately measuring an ''integrated concentration'' without having to manually draw intermittent samples [20–22]. Sprague–Dawley rats weighting 300–380 g were used. For the automated blood sampling experiments, the rats were implanted with a jugular vein catheter (CX-2010, BAS) and/or femoral vein catheter (CX-2020, BAS). After surgery, the rats were installed in the Raturn, then allowed to recover for 1 day with free access to food and water. The rats were dosed intraperitoneally (i.p.), and intravenously (i.v.) with EGCG solution prepared in physiological saline. The blood was automatically withdrawn from the jugular vein and followed by a heparin/saline flush. A total 150 μl of blood–saline (1:1) was collected by the fraction collector at each time point.

3. Results and discussion

3.1. Method development

Liquid chromatography–electrochemistry with multi-electrode detection has proven useful in the identification and determination of phenolic compounds [23–25]. Monitoring four potentials simultaneously gave a better voltammetric characterization of EGCG in plasma. Peak assignment and purity can be assured by the comparing the ratios at different energies between standard and samples [26].

The mobile phase was 20 m*M* monochloroacetatic acid, pH 2.8 containing 12% (v/v) acetonitrile. The pump flow-rate was 1.2 ml/min.

3.2. Validation of assay

3.2.1. Selectivity

Chromatograms were obtained and compared between the blank plasma and plasma containing EGCG (Fig. 1) at +600 mV vs. Ag/AgCl applied voltage. No interfering peaks were detected at the retention times of EGCG. Samples could be injected every 10 min.

3.2.2. Linearity

A weighed linear regression of the peak height versus standard concentrations was performed for EGCG using a mass of 1/concentration. The observed peak heights were linear over the concentration range of 5–800 ng/ml in rat plasma. The mean values (±SD) for slope, intercept and r^2 were 0.96±0.05, 17.25±11 and 0.9984±0.001, respectively, for three calibration curve plots.

3.2.3. Limit of detection and quantitation

The detection limit of EGCG in rat plasma was determined at 2 ng/ml with a signal-to-noise ratio of 3. The limit of quantitation was 5 ng/ml.

3.2.4. Accuracy and precision

The intra- and inter-day accuracy and precision values for QC samples are provided in Table 1. The precision values (RSD) at the three concentrations in the intra-assay study varied between 1.3 and 4.5% and in the inter-assay study varied between 2.2 and 4.4%. The accuracy (% bias) values for all three

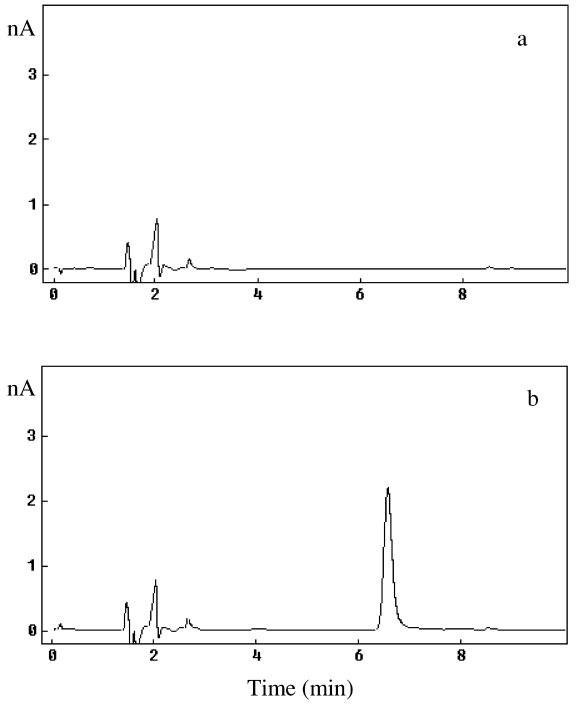

Fig. 1. Chromatograms of extract from (a) blank rat plasma and (b) real blood sample (384 ng/ml) draw after i.v. administration of EGCG at a dose of 1 mg/kg to rat. Applied potential: +600 mV vs. Ag/AgCl.

concentrations deviated less than 6.5% from the corresponding nominal concentrations.

3.2.5. Extraction recovery and stability

A comparison of neat standard versus plasma-extracted standard indicated that the extraction recovery of the analyte from rat plasma was 78%.

Table 1
Accuracy and precision for EGCG assay in rat plasma

Concentration added (ng/ml)	Concentration measured (mean±SD) (ng/ml)	RSD (%)	Bias (%)
Intra-day (n=3)			
50	48.3±2.2	4.5	−3.4
200	187±2.4	1.3	−6.5
400	412±11	2.7	2.8
Inter-day (n=3)			
50	48.7±1.9	3.9	−2.6
200	190±8.3	4.4	−5.0
400	401±8.7	2.2	0.3

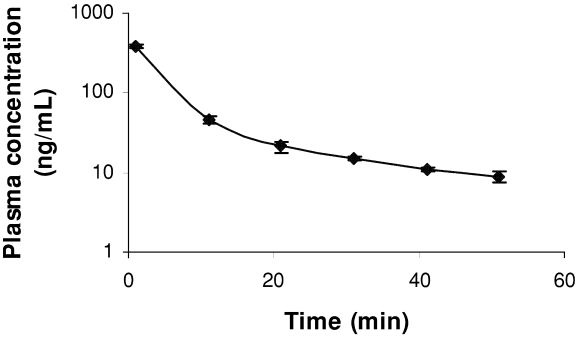

Fig. 2. Mean (±SD) plasma concentration versus time profile of EGCG in rats (n=3) following a single 1 mg/kg intravenous administration.

EGCG was stable in rat blood in the dark and 4°C for 6 h. As far as the stability of processed samples after extraction was concerned, no significant loss of EGCG was observed at 4°C for 10 h.

3.3. Pharmacokinetic results

The proposed method was used for the determination of EGCG in rat plasma. Fig. 2 illustrates data for a single 1 mg/kg intravenous dose administration of EGCG to rats (n=3). Fig. 3 illustrates data for a single 2 mg/kg intraperitoneal dose administration of EGCG to rats (n=4). The pharmacokinetic parameters derived from intravenous and intraperitoneal administration are summarized in Table 2. This

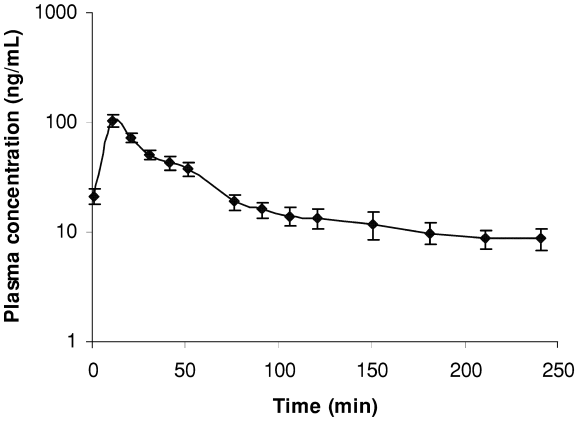

Fig. 3. Mean (±SD) plasma concentration versus time profile of EGCG in rats (n=4) following a single 2 mg/kg intraperitoneal administration.

Table 2
Pharmacokinetic parameters of administration of EGCG in rat

i.v. administration (1 mg/kg)			i.p. administration (2 mg/kg)			
K_{el} (min^{-1})	$t_{1/2}$ (min)	AUC (ng min/ml)	C_{max} (ng/ml)	t_{max} (min)	K_{ab} (min^{-1})	K_{el} (min^{-1})
0.46±0.04	1.55±0.14	5147±34	104.4±12.4	11±2.5	0.11±0.02	0.04±0.007

method is being used to study EGCG kinetics using various routes of administration to better understand the potential role of this compound in human consumption of green tea.

4. Conclusion

A four-channel liquid chromatography–electrochemistry procedure was developed and evaluated for the determination of EGCG in rat plasma. The blood sampling robot and the reported method offer several advantages, such as accurate withdrawal of blood with low animal stress, a rapid and clean extraction scheme and a short chromatographic run time.

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