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Journal of Chromatography A, 1032 (2004) 125–128
JOURNAL OF CHROMATOGRAPHY A
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Determination of (−)-epigallocatechin gallate in rat blood by microdialysis coupled with liquid chromatography

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Abstract

A liquid chromatographic method coupled with microdialysis was used to determine the protein-unbound (−)-epigallocatechin-3-gallate (EGCG) in rat blood. EGCG and dialysates were separated using a Merck RP-18e column maintained at ambient temperature, and a mobile phase comprised of acetonitrile-10 mM monopotassium phosphate (pH 3.82) (20:80, v/v) with a flow rate of 1.0 ml/min. The UV detector wavelength was set at 206 nm. The detection limit for EGCG was 10 ng/ml. The concentration-response relationship was linear ($r^2 > 0.995$) over a concentration range of 0.05–10 μg/ml; intra- and inter-assay precision and accuracy of EGCG fell within predefined limits. Pharmacokinetic parameters of EGCG were assessed using compartmental models. The disposition of EGCG in the rat blood suggests that EGCG was fitted by two-compartmental model. The distribution and elimination half-lives were 6 and 72 min respectively, after the dosage of 30 mg/kg.

Keywords: Microdialysis; Epigallocatechin gallate; Catechins

1. Introduction

Tea is one of the most popular beverages in the world. (−)-Epigallocatechin-3-gallate (EGCG; Fig. 1) is the major polyphenol in tea, containing approximately 50% of the catechins in green tea [1]. EGCG has been found to possess antioxidant activity and is used as topical skin cancer prevention [2]. In addition, EGCG is antibacterial [3], inhibits human immunodeficiency virus [4], reduces platelet aggregation [5], and prevents the development of atherosclerosis [6]. It has also been reported that EGCG may be closely related to the preventive effects on inflammation caused by leukocyte elastase inhibition [7]. In order to investigate the pharmacokinetics of EGCG, several analytical methods have been reported to measure its concentration in biological fluids such as liquid chromatography (LC) with UV [8], LC with electrochemical detection [9,10], LC with chemiluminescence [11], and LC with electrospray mass spectrometry [12].

Although numerous studies describe the pharmacokinetics [13] and measurement [14] of EGCG in biological fluids, no researchers have examined the protein-unbound form of EGCG in blood yet. The aim of this current investigation is to use microdialysis to study the pharmacokinetics of EGCG in free form after a single i.v. bolus administration in rats. For this, a microdialysis probe was inserted into the jugular vein for continual blood sampling and analysis by HPLC.

2. Methods

2.1. Animals

Male specific pathogen-free Sprague–Dawley rats were obtained from the Laboratory Animal Center of the National Yang-Ming University, Taipei. The animals had free access to food (Laboratory rodent diet No. 5P14, PMI Feeds, Richmond, IN, USA) and water until 18 h prior to being used in experiments, at which time only food was removed. Six Sprague–Dawley rats (280–320 g) were initially anesthetized with urethane 1 g/ml and α-chloralose

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doi:10.1016/j.chroma.2003.08.077

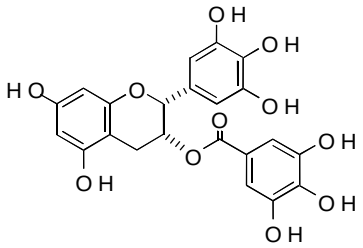

Fig. 1. Chemical structure of epigallocatechin gallate (EGCG).

0.1 g/ml (1 ml/kg, i.p.), and remained anesthetized throughout the experimental period. The femoral vein was exposed for further drug administration. The rat's body temperature was maintained at 37 °C with a heating pad.

2.2. Chromatography

HPLC-grade solvents and reagents were obtained from Merck (Darmstadt, Germany). Three-fold deionized water (Millipore, Bedford, MA, USA) was used for all preparations. The HPLC system consisted of a chromatographic pump (BAS PM-80, West Lafayette, IN, USA), an injector (Rheodyne 7125, Cotati, CA, USA) equipped with a 20 μl sample loop, and an ultraviolet detector (Varian, Walnut Creek, CA, USA). EGCG and dialysates were separated using a Merck RP-18e column (250 mm × 4.6 mm i.d.; particle size 5 μm) and its guard column (10 mm × 4.6 mm i.d.) maintained at ambient temperature. The mobile phase was comprised of acetonitrile-10 mM monopotassium phosphate (pH 3.82) (20:80, v/v), and the flow rate of the mobile phase was 1 ml/min. The buffer was filtered through a Millipore 0.45 μm filter and degassed prior to use. The UV detector was set at 206 nm and connected to an EZChrom chromatographic data system (Scientific Software, San Ramon, CA, USA).

2.3. Method validation

All calibration curves were required to have a correlation value of at least 0.995. The intra- and inter-assay variabilities were determined by quantitating six replicates on the same day and six consecutive days, respectively (Table 1). The accuracy (bias) was calculated from the nominal concentration (C_{nom}) and the mean value of observed concentrations (C_{obs}) as follows: bias(%) $= [(C_{obs} - C_{nom})/C_{nom}] \times 100$. The precision, expressed as relative standard deviation (R.S.D), was calculated from the observed concentrations as follows: R.S.D (%) = [standard deviation (S.D.)/C_{obs}] × 100. To avoid degradation, the EGCG storage solution contained ascorbic acid (20%, w/v) and EDTA (0.1%, w/v) [15].

2.4. Blood microdialysis

Blood microdialysis systems comprised of a CMA/100 microinjection pump (CMA, Stockholm, Sweden) and microdialysis probe. The dialysis probes for blood (10 mm in length) were made of silica capillary in a concentric design [16]. Their tips were covered by dialysis membrane (Spectrum Co., 150 μm outer diameter with a cut-off at nominal molecular mass of 18 000, Laguna Hills, CA, USA) and all unions were cemented with epoxy. At least 24 h was allowed for the epoxy to cure. The blood microdialysis probe was located within the jugular vein/right atrium and then perfused with anticoagulant dextrose (ACD) solution (citric acid 3.5 mM; sodium citrate 7.5 mM; dextrose 13.6 mM) at a flow rate of 2 μl/min employing the microinjection pump [16].

Table 1
Intra- and inter-assay precision (RSD) and accuracy (bias) of the HPLC method for the determination of EGCG

Nominal concentration (g/ml)	Observed concentration (g/ml)	R.S.D (%)	Bias (%)
Intra-assay			
0.05	0.048 ± 0.004	8.3	−4.0
0.10	0.097 ± 0.006	6.2	−3.0
0.50	0.499 ± 0.003	0.6	−0.02
1.00	1.003 ± 0.019	1.9	0.3
5.00	5.058 ± 0.094	0.1	1.2
10.00	9.936 ± 0.221	2.2	−0.6
Inter-assay			
0.05	0.051 ± 0.005	9.8	2.0
0.10	0.096 ± 0.005	5.2	−0.4
0.50	0.501 ± 0.005	1.0	0.2
1.00	1.025 ± 0.029	2.8	2.5
5.00	4.949 ± 0.109	2.2	−1.0
10.00	9.909 ± 0.285	2.9	−0.9

[a] Data expressed as means ± S.D. ($n = 6$).

Outflows from blood microdialysates were connected to a fraction collector (CMA/140) every 10 min and the same chromatographic system was subsequently analyzed following blood dialysate. A retrograde calibration technique was utilized during in vivo recovery. The blood microdialysis probes were inserted into the rats' jugular vein under anesthesia with urethane and α-chloralose. Following a stabilization period of 2 h post probe implantation, the perfusate (C_{perf}) and dialysate (C_{dial}) concentrations of EGCG were determined by HPLC. The ACD solution (for blood microdialysis) containing EGCG was perfused through the probe at a constant flow rate (2 μl/min) employing the infusion pump (CMA/100). The in vivo relative recovery (R_{dial}) of EGCG across the microdialysis probe was calculated by the following equation: $R_{dial} = (C_{perf} - C_{dial})/C_{perf}$. The microdialysate recovery and concentration calculations were performed according to our previous report [16]. EGCG microdialysate concentrations (C_m) were converted to unbound concentration (C_u) as follows: $C_u = C_m/R_{dial}$.

2.5. Drug administration

After a 2 h post-surgical stabilization period of probe implantation, EGCG (30 mg/kg; Sigma, St. Louis, MO, USA)

was i.v. bolus administered. Six individual animals were used in the experiment. The sampling interval was 10 min for each probe. Blood dialysates were measured by HPLC in the same experimental day.

2.6. Pharmacokinetics and data analysis

Pharmacokinetic calculations were performed on each individual animal's data utilizing the pharmacokinetic calculation software WinNonlin Standard Edition Version 1.1 (Scientific Consulting, Apex, NC, USA) by a compartmental method. The results are represented as mean ± standard deviation of the mean.

3. Results and discussion

Under the conditions described above, the retention time of EGCG was found to be 5.35 min (Fig. 2). Fig. 2A shows a standard injection of EGCG (5.0 μg/ml), and Fig. 2B shows the chromatogram of a blank blood dialysate. None of the observed peaks interfered with the analyte. Fig. 2C shows the chromatogram of a blood dialysate sample containing EGCG (2.79 μg/ml) collected 10 min after EGCG administration (30 mg/kg, i.v.).

The calibration curve of EGCG was obtained prior to HPLC analysis of dialysates over concentration ranges of 0.05–10 μg/ml. The concentration of EGCG was linearly related to the peak areas in the chromatogram ($r^2 > 0.995$). The detection limit of EGCG was 10 ng/ml at a signal-to-noise ratio of 3.

As shown in Table 1, the overall mean precision, as defined by the RSD, ranged from 0.6 to 9.8%. Analytical accuracy, expressed as the bias, varied from −4.0 to 2.5%. Thus, the intra- and inter-assay accuracy and precision were found to be acceptable for the analysis of a dialysis sample in support of pharmacokinetic studies. The average in vivo recovery ($n = 3$) of EGCG in blood at concentrations of 1, 2, and 5 μg/ml were 0.41 ± 0.04, 0.44 ± 0.01, and 0.42 ± 0.01 Hence, actual concentration of EGCG in rat blood could be corrected by the average recovery of 0.42. Unbound drug concentration data obtained from the microdialysis technique are usually transferred by recovery. The fact that mass transport passes the dialysis membrane is governed by diffusion and managed by the concentration gradient. The microdialysis recovery is independent of outside substance concentration, thus, monitoring the substance concentrations increased or decreased outside the membrane were obtainable.

The concentration versus time curve of EGCG in rat blood indicating that disposition of EGCG in rat blood has a slower and longer elimination phase. The pharmacokinetic models (one- versus two-compartment) were compared according to the criteria of Akaike information criterion (AIC) [17], with minimum AIC (Akaike information criterion) values being regarded as the best representation of the blood concentration-time course data. A compartment model with individual animal data after dose was proposed by the computer program WinNonlin. This AIC value decreases from −20.07 for one-compartment model to −51.91 for the two-compartment model, indicating that the two-compartment model is better than the one-compartment model. The following equation applies to a two-compartment pharmacokinetic model: $C = Ae^{-\alpha t} + Be^{-\beta t}$; where A and B are the concentrations (C), of intercept for fast and slow disposition phases, re-

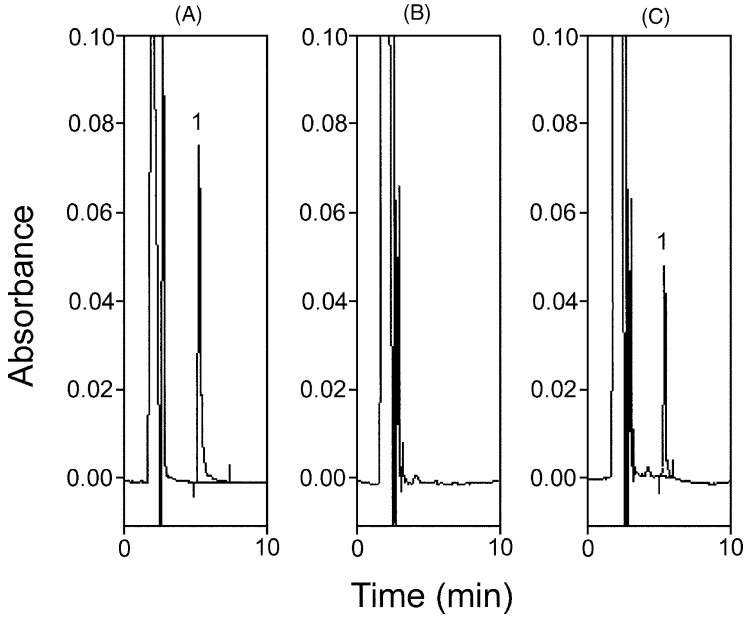

Fig. 2. Typical chromatograms of (A) standard epigallocatechin gallate (EGCG) (5 μg/ml), (B) blank blood dialysate from the microdialysis probe before drug administration, and (C) blood dialysate sample containing EGCG (2.79 μg/ml) collected 10 min after EGCG administration (30 mg/kg). 1: EGCG.

Table 2
Pharmacokinetic parameters of EGCG in rats after 30 mg/kg administration

Parameters	Estimated
A (μg/ml)	23.13 ± 14.93
B (μg/ml)	0.81 ± 0.61
α (1/min)	0.14 ± 0.053
β (1/min)	0.02 ± 0.017
$t_{1/2,\alpha}$ (min)	6 ± 3
$t_{1/2,\beta}$ (min)	72 ± 82
AUC (μg min/ml)	190 ± 57
Vss (ml/kg)	5636 ± 6480
Cl (ml/min/kg)	172 ± 57

Data expressed as means ± S.D. ($n = 6$). $t_{1/2,\alpha}$: distribution half-life; $t_{1/2,\beta}$: elimination half-life; AUC: area under the concentration versus time curve; Vss: volume of distribution at steady-state; Cl: clearance.

spectively; and α and β are the disposition rate constants for fast and slow disposition phases, respectively. Data analysis after i.v. injection of EGCG at 30 mg/kg yields the following equation $C = 23.13e^{-0.14t} + 0.81e^{-0.02t}$. The pharmacokinetic parameters as derived from these data and calculated by WinNonlin program are shown in Table 2.

The pharmacokinetics of EGCG in total blood concentration has also been reported to be characterized by bi-exponential disposition [13]. Compared to methods of biological fluids sampling and sample clean-up procedures, microdialysis provides a powerful sampling system for small animals, requiring no loss of biological fluids, and providing higher temporal resolution than traditional total blood sampling techniques. Instead of the discrete time point sampling method of traditional total blood sampling techniques, microdialysis makes possible continuous sampling.

In conclusion, we have developed a specific, endogenous interference-free and economical microdialysis sampling method for the determination of protein-unbound EGCG in rat blood. The data obtained suggest that EGCG was fitted well by a two-compartmental pharmacokinetic model.

Acknowledgements

This study was supported in part by research grants from the National Science Council (NSC91-2113-M-077-008; NSC91-2320-B-077-012), Taiwan.

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