Excretion of Four Catechins in Tea Polyphenols in Rats

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Abstract: **Objective** To investigate excretion profiles of the four major anti-oxidant active catechins, (–) epigallocatechin-3-gallate (EGCG), (–) epicatechin-3-gallate (ECG), (–) epigallocatechin (EGC), and epicatechin (EC) in tea polyphenols (TP) in rats in order to provide experimental data for clinical uses and development of TP as a novel drug. **Methods** The above four catechins in urine, bile, and feces were simultaneously determined by high performance liquid chromatography coupled with ultraviolet absorption detector (HPLC-UV) assay with a binary gradient elution. The samples were extracted by ethyl acetate prior to HPLC. The quantification was carried out by peak area internal standard method. Following iv dosing TP 100 mg/kg to rats, the samples were collected at different time intervals up to 8 h (urine and bile) and 24 h (feces). **Results** The urinary $A_{e,\ 0\text{-}8\ h}$ (cumulative excretion amount over 8 h) of EGCG, ECG, EGC, and EC were, on the average, 150.83, 30.75, 116.69, and 254.56 μg, corresponding to $f_{e,\ 0\text{-}8\ h}$ (cumulative excretion fraction of dose over 8 h) of 1.45%, 0.84%, 7.88%, and 10.73%, respectively; the biliary $A_{e,\ 0\text{-}8\ h}$ were 12.61, 42.64, 6.61, and 1.24 μg, corresponding to the $f_{e,\ 0\text{-}8\ h}$ of 0.12%, 1.16%, 0.45%, and 0.053% , respectively. For fecal excretion, only EGCG and EGC were detected with $A_{e,\ 0\text{-}24\ h}$ of 7.38 μg ($f_{e,\ 0\text{-}24\ h}$ of 0.07%) and 157 μg ($f_{e,\ 0\text{-}24\ h}$ of 9.99 %), respectively. The $f_{e,\ total}$ (the total f_e of 3 excretory routes) were 18.32%, 10.78%, 2.00%, and 1.64% for EGC, EC, ECG, and EGCG, respectively. **Conclusion** EGCG and EC are mainly excreted in urine, ECG in bile, and EGC in feces by reference to their A_e and f_e. The excretion of the four catechins based on $f_{e,\ total}$ is ranked in order of EGC > EC > ECG > EGCG. Only small amount of four catechins are recovered in urine, bile, and feces, indicating an extensive metabolic conversion of catechins in the rat body.

Key words: epicatechin; (–) epicatechin-3-gallate; (–) epigallocatechin; (–) epigallocatechin-3-gallate; excretion; tea polyphenols
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Introduction

Tea (*Camellia sinensis* O. Ktze., Theaceae), a well-known popular beverage around the world, especially in China, has long been proposed to have a wide range of beneficial health effects, including chemo-prevention of cancers, protection of organs from injuries induced by ischemia-reperfusion, lowering of blood lipid and glucose level, reducing of obesity, etc (Yang, Chung, and Yang, 2000; Lambert and Yang, 2003; Lou *et al*, 1998; Murase *et al*, 2002). These effects have been attributed mostly to the tea polyphenols (TP) present in tea, commonly known as tea catechins, the major ones being (–) epigallocatechin-3-gallate (EGCG), (–) epicatechin- 3-gallate (ECG), (–) epigallocatechin (EGC), and epicatechin (EC) (Fig. 1), of which EGCG is the most abundant and active, ECG next (Graham, 1992; Luo *et al*, 2005; Hashimoto *et al*, 1999).

In the past decade, our team was devoted to the research on pharmacology of TP, and found that TP had very potent anti-free radical activity and powerful health-promoting effects (Wang *et al*, 2006; Lü *et al*, 2007a; Lü *et al*, 2007; Lü *et al*, 2007b). More recently, we started pharmacokinetic studies of TP in animals by means of a reversed-phase high performance liquid

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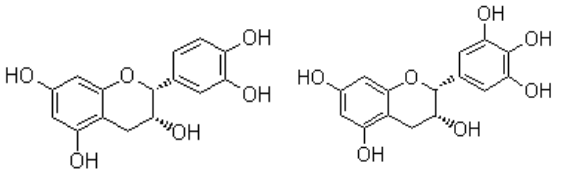
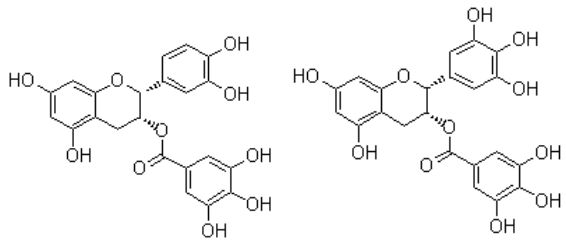
chromatography coupled with ultraviolet absorption detector (RP-HPLC-UV) assay for simultaneous determination of catechins in rat plasma (Fu *et al*, 2008). To date, the excretion of tea catechins in urine, bile, and feces has not well been characterized, though available four information on plasma pharmacokinetics and tissue distribution of TP (Chen *et al*, 1997; Kim *et al*, 2000; Lin *et al*, 2007; Li *et al*, 2001). In the present article, we report the excretory profiles of above mentioned catechins in rats receiving iv administration of TP in order to provide useful data for its clinical rational uses and development as a novel drug.



(–) epicatechin (EC)
(–) epigallocatechin (EGC)



(–) epicatechin-3-gallate (ECG)
(–) epigallocatechin-3-gallate (EGCG)

Fig. 1 Chemical structures of four major catechins present in green tea

Materials and methods

Chemical reagents

TP, as an extract from green tea, was purchased from Wuyuan Tea Plantation (Jiangxi Province, China) with the purity > 98%, determined by spectrophotometry with ferrous tartrate as chromogenic agent, and the percent content of 46.81% EGCG, 16.54% ECG, 6.64% EGC, and 10.60% EC, respectively, measured by the HPLC-UV assay in our laboratory, and formulated as injection with sterile water for experimental use. EGCG, ECG, and EGC reference substances were purchased from U-sea Biotech Co., Ltd (Shanghai, China) and with purity of 99.0%; EC reference substance was provided by China Institute for Control of Pharmaceutical & Biological Products and with purity of 97%; Vanillin as internal standard (IS) was purchased from Kermel Chemical Reagent Co., Ltd. (Tianjin, China) with purity of 98%; Acetonitrile (CH_3CN), methanol (MeOH), all of HPLC grade, were purchased from Tedia Company (USA); Ethyl acetate (EtOAc), Vitamine C, and all other chemicals used were commercially available highest grade; The double-distilled water was used for preparing mobile phase and other solutions.

Chromatographic condition

The HPLC was performed using an Agilent 1100 series LC system equipped with a quaternary pump, an online degasser, a UV-detector, a refrigerated autosampler. The system control and data processing were carried out by a Chemstation 32 software. The chromatographic separation was achieved on a Kromasil-C_{18} analytical column (150 mm × 4.6 mm, 5 μm) protected by a Chromasil-C_{18} pre-column (20 mm × 4.6 mm, 5 μm) (Ilite, Dalian, China) maintained at room temperature using a mobile phase composed of CH_3CN (A) and 0.1% citric acid (B). The column was eluted with a linear gradient: 0 min (16% A, 1.0 mL/min)→14 min (20% A, 1.0 mL/min)→18 min (16% A, 1.5 mL/min)→22 min (16% A, 1.0 mL/min) for urine and bile samples and 0 min (13% A, 1.0 mL/min)→8 min (13% A, 1.3 mL/min)→19 min (19% A, 1.8 mL/min)→27 min (13% A, 1.0 mL/min) for feces samples. The eluate was monitored at 280 nm.

Experimental animals

Male SD rats weighing 250–300 g were obtained from Animal Center of Dalian Medical University and housed in standard laboratory conditions and allowed free access to water and commercial rat chow. They were maintained under a constant 12 h light-dark cycle at an environment temperature of 21–23 ℃. All animal experiments were carried out in accordance with institutional guidelines and ethics. Rats were injected *via* tail vein with TP injection at a dose of 100 mg/kg,

equivalent to 46.87 mg EGCG, 16.62 mg ECG, 6.64 mg EGC, and 10.60 mg EC per kg BW. After dosing, urine and feces samples were collected into ice-cold tubes from five conscious rats individually placed in metabolic cages at different time intervals; bile samples were collected into ice-cold tubes from five bile duct-cannulated rats under anesthesia with urethane (1.0 g/kg) ip. The above collected samples were stored at −20 ℃.

Sample preparation

An aliquot of 200 μL diluted urine and bile (5-fold dilution preferred) samples and 100 μL of 1 g∶10 mL feces homogenate in MeOH were transferred into an Eppendorf tube containing 20 μL each of 10 μg/mL IS and 20% Vitamin C solution and then extracted twice with 400 μL of EtoAc each by vortex-mixing for 0.5 min and centrifuged at 3 000 × *g* for 10 min at 4 ℃. The upper organic phase was transferred into another tube and evaporated to dry under a gentle N_2 stream at 37 ℃ in a water bath. The residue was reconstituted in 100 μL of a 20% CH_3CN aqueous solution. After centrifuging at 3 000 × *g* for 10 min at 4 ℃, 50 μL of supernatant was injected into chromatograph for HPLC analysis.

Quantification of the four catechins in urine, bile, and feces samples of rats

The frozen urine, bile, and feces samples of rats were thawed naturally at ambient temperature and then treated as described above. Then, peak area ratio of analyte/IS was calculated with reference to the recorded chromatogram. Samples were quantified by reference to accompanying calibration extraction curves of parent catechins produced by the processing and analysis of blank rat urine, bile, and feces to which appropriate quantities of EGCG, ECG, EGC, and EC standards had been added and constructed by potting the peak area ratios of each analyte/IS *vs* analyte concentration in urine, bile, and feces homogenate standard samples.

Validation of methodology

This HPLC assay was validated thoroughly in terms of specificity, linearity, precision, accuracy, stability, and extraction recovery according to the *Guiding Principles* issued by China SFDA (Zheng, 2002). The quality control (QC) samples containing appropriate amount of EGCG + ECG + EGC + EC, prepared based on procedures described under section sample preparation, at high, middle, and low concentration for five repeated analysis separately, were used for validation.

Data analysis

Data from individual rat were used to calculate the mean concentration for EGCG, ECG, EGC, and EC at each time interval (0–0.5, 0.5–2, 2–4, 4–6, and 6–8 h for urine and bile; 0–8 and 8–24 h for feces). The excretion parameters were calculated as follows:

X (amount excreted in each time interval, μg) = V (volume, mL) × C (concentration, μg/mL) × N (dilution multiple)

A_e (cumulative amount excreted up to any time, μg) = the sum of all drug excreted up to that time

excretion rate (μg/h) = A_e (μg) / τ (collection time interval, h)

f_e (cumulative excretion fraction of dose) = A_e (μg) / [BW (kg) × dose of TP (mg/kg) × percent content of each constituent in TP (%) × 10^3]

$A_{e,\ total}$ (total cumulative excretion amount of three excretory routes) = the sum of $A_{e,\ urine} + A_{e,\ bile} + A_{e,\ feces}$

$f_{e,\ total}$ (total cumulative excretion fraction of three excretory routes) = the sum of $f_{e,\ urine} + f_{e,\ bile} + f_{e,\ feces}$

Statistical analysis was performed by a Student's *t*-test to identify significant differences between mean values of two groups. All data were expressed as mean ± SD. $P < 0.05$ was considered to be statistically significant. The software SPSS version 11.5 was used for calculations.

Results

Validation of methodology

The Fig. 2 and Fig. 3 illustrated that under specified HPLC and sample preparation conditions, EGCG, ECG, EGC, EC, and IS were well resolved from each other without interference from endogenous substances, metabolites, and impurities of TP raw material. The calibration curves of urine / bile / feces had good linearity over the range of 0.5–10, 0.2–5, and 1–25 μg/mL for EGCG, 0.25–2.5, 0.2–5, and 0.8–20 μg/mL for ECG, 2.5–50, 0.8–20, and 4–100 μg/mL for EGC,

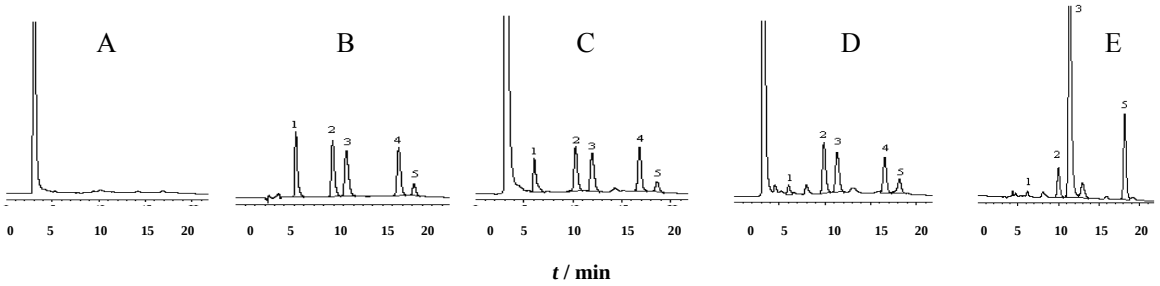


Fig. 2 Representative chromatogram of urine sample

1. EGC (t_R 6.21 min); 2. EC (t_R 10.42 min); 3. EGCG (t_R 11.81 min); 4. IS (t_R 16.36 min); 5. ECG (t_R 18.02 min)

A. blank urine; B. reference substances and internal standard; C. blank urine spiked with reference substances and internal standard; D. urine sample from a rat after iv administration of 100 mg/kg dose of TP; E. TP injection (diluted to 50 μg/mL)

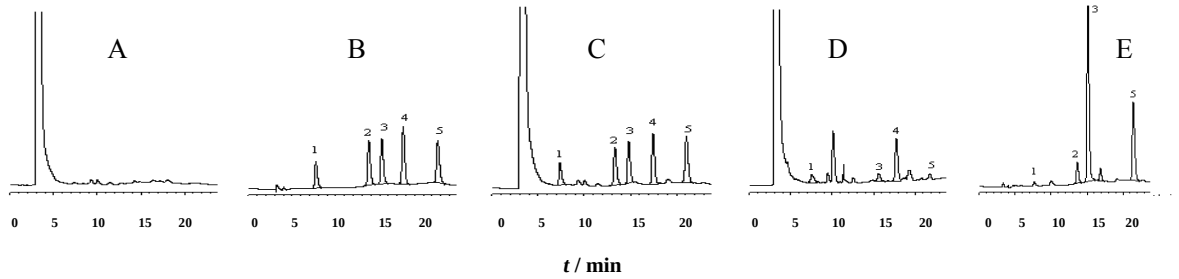


Fig. 3 Representative chromatogram of feces sample

1.EGC (t_R 7.86 min); 2.EC (t_R 13.59 min); 3.EGCG (t_R 15.34 min); 4.IS (t_R 17.44 min); 5.ECG (t_R 21.73 min)

A. blank feces; B. reference substances and internal standard; C. blank feces spiked with reference substances and IS; D. feces sample from a rat after iv administration of 100 mg/kg dose of TP; E. TP injection (diluted to 50 μg/mL)

and 1–20, 0.4–10, and 2–50 μg/mL for EC ($r^2 > 0.995$), respectively. The intra-day and inter-day precision (RSD) of QC samples was better than 10%, and average accuracy was between 90%–110%. The extraction recovery of the four catechins and IS was better than 60% (urine), 80% (bile), and 70% (feces). The samples of urine, bile, and feces were stable for at least 8 h at room temperature, 12 h at 4 ℃ and 30 d at −20 ℃.

Excretion of four catechins in urine

As seen from Fig. 4, $A_{e,\ urine}$ of EGCG, ECG, EGC, and EC had reached plateau at 8 h post dose, and were (150.83 ± 14.20), (30.75 ± 2.81), (116.69 ± 45.08), and (251.97 ± 46.07) μg over 8 h, respectively, corresponding to the $f_{e,\ 0\text{-}8\ h}$ of 1.45%, 0.84%, 7.88%, and 10.62% (Table 1). Based on both $A_{e,\ 0\text{-}8\ h}$ and $f_{e,\ 0\text{-}8\ h}$, EC was most significantly recovered in urine relative to other three catechins ($P < 0.01$). The four catechins were detectable in the earliest urine fraction collected (up to 0.5 h), as illustrated in Fig. 4 and Fig. 5; furthermore, the $A_{e,\ 0\text{-}0.5\ h}$ of EGCG, ECG, EGC, and EC represented 40.70%, 49.60%,

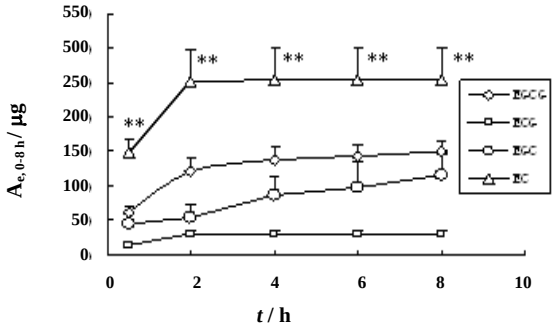


Fig. 4 Cumulative urinary excretion of the four catechins in rats receiving iv administration of TP 100 mg/kg (n = 5)

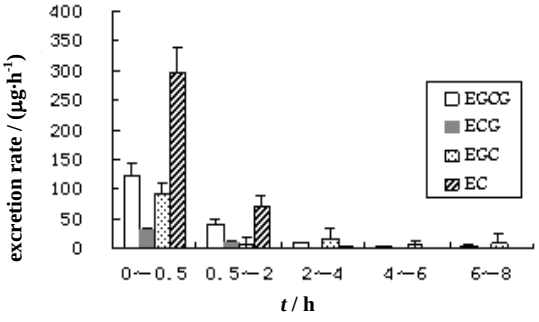


Fig. 5 Urinary excretion rate of the four catechins in rats receiving iv administration of TP 100 mg/kg (n = 5)

Tabel 1 Excretion of four catechins in urine, bile, and feces of rats receiving iv administration of TP 100 mg/kg (*n*=5)

Samples	A_e / μg				f_e / %			
	EGCG	ECG	EGC	EC	EGCG	ECG	EGC	EC
Urine(0–8 h)	150.83±14.20	30.75±2.81	116.69±45.08	251.97±46.07**	1.45±0.18	0.84±0.10	7.88±2.89	10.62±1.36**
Bile(0–8 h)	12.61±1.69	42.64±9.53**	6.61±1.82	1.24±0.83	0.12±0.01	1.16±0.24**	0.45±0.12	0.053±0.04
Feces(0–8 h)	7.38±3.34	–	118.09±29.56**,▲	–	0.07±0.03	–	7.55±2.01**,▲	–
Feces(0–24 h)	7.38±3.34	–	157.13±60.82**,▲	–	0.07±0.03	–	9.99±3.75**,▲	–
total	170.82±19.23	73.39±12.34	280.43±107.7*,△	253.21△±46.83	1.64±0.22	2.00±0.25	18.32±6.76*,△	10.67±1.40△

*$P < 0.05$, **$P < 0.01$ *vs* other three catechins

△$P < 0.05$ *vs* corresponding ester-type catechins

▲$P < 0.01$ *vs* bile

38.45%, and 58.15% of their $A_{e,\ 0\text{-}8\ h}$, respectively. In addition, the urinary excretion rate was the biggest within 0–0.5 h post dose for all four catechins, and then declined rapidly; after 2 h four catechins were recovered in negligible amount (Fig. 5).

Excretion of four catechins in bile

The $A_{e,\ bile}$ of EGCG, ECG, EGC, and EC had also reached plateau at 8 h post dose, as shown in Fig. 6; with $A_{e,\ 0\text{-}8\ h}$ being (12.61 ± 1.69), (42.64 ± 9.53), (6.61 ± 1.82), and (1.24 ± 0.83) μg, corresponding to $f_{e,\ 0\text{-}8\ h}$ of 0.12%, 1.16%, 0.45%, and 0.053%, respectively (Table 1). In contrast to urinary excretion, ECG, instead of EC, was the largest in both $A_{e,\ 0\text{-}8\ h}$ and $f_{e,\ 0\text{-}8\ h}$ of bile ($P < 0.01$). EGC and EGCG were detectable only in the first and second fractions collected up to 2 h; EC showed the poorest biliary excretion ability. Fig. 7 illustrated that the biliary excretion rate was the biggest at 0 – 0.5 h for four catechins; The $A_{e,\ 0\text{-}0.5\ h}$ of EGCG, ECG, EGC, and EC accounted for 27.90%, 13.57%, 63.91%, and 65.43% of $A_{e,\ 0\text{-}8\ h,}$ respectively; ECG among the four catechins had the largest excretion rate and was recovered up to 8 h.

Excretion of four catechins in feces

From Table 1, it was found that unlike urine and bile, EGC predominated in feces, with $A_{e,\ 0\text{-}8\ h}$ and $A_{e,\ 0\text{-}24\ h}$ being (118.09 ± 29.56) and (157.13 ± 60.82) μg as well as $f_{e,\ 0\text{-}8\ h}$ and $f_{e,\ 0\text{-}24\ h}$ being 7.55% and 9.99%, respectively. EGCG showed very insignificant excretion by this route, as reflected by a very low $A_{e,\ 0\text{-}24\ h}$ (7.38 μg) and $f_{e,\ 0\text{-}24\ h}$ (0.07%). It was also evident that the other two catechins (ECG and EC) were undetectable in feces.

Total f_e of urine, bile, and feces ($f_{e,\ total}$) for four catechins

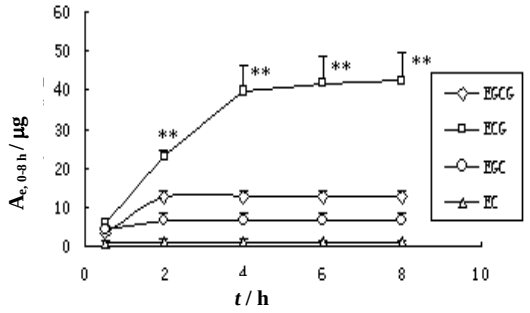


Fig. 6 Cumulative biliary excretion of the four catechins in rats receiving iv administration of TP 100 mg/kg (*n* = 5)

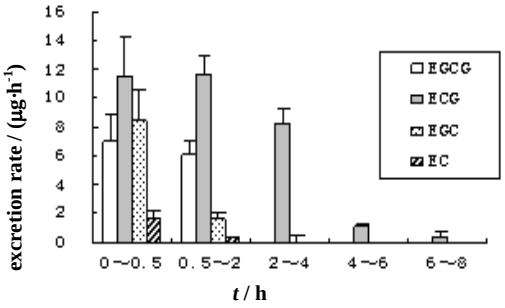


Fig. 7 Biliary excretion rate of the four catechins in rats receiving iv administration of TP 100 mg/kg (*n* = 5)

It was apparent from Table 1 that the $f_{e,\ total}$ for the four catechins was ranked in descending order of EGC > EC > ECG > EGCG, showing that the non-gallic acid ester type catechins (EGC and EC) had higher $f_{e,\ total}$ than corresponding gallic acid ester type catechins (EGCG and ECG) ($P < 0.05$). In addition, it was found based on A_e and f_e that EGCG and EC were excreted mainly *via* urine, ECG mainly *via* bile, EGC mainly *via* feces.

Discussion

In this paper, we described an HPLC-UV assay, which

employed a binary gradient elution and thus yielded a high specificity, enabling good resolution between four catechins and IS, and no interference from matrix, metabolites, and impurities in TP raw material. For urine and bile samples the same HPLC conditions and sample preparation procedures were used, whereas for feces samples where much more interferences were present in the matrix, a different but improved chromatographic condition was performed. This HPLC assay developed by us resulted in a single run time of 20 min, which was much shorter than that of 30 min for initially developed isocratic elution where mobile phase composed of CH_3CN–0.1% citric acid (14∶86) and a flow rate of 1.5 mL/min were used (Fu *et al*, 2008).

In this study, we utilized IS method, instead of external standard method as reported in most currently available information on HPLC assay of tea catechins in biological samples (Chen *et al*, 1997; Kim *et al*, 2000), to analyze the four catechins in urine, bile, and feces. We chose Vanillin as IS from nearly 20 chemicals, it was not only similar to catechin in chemical structure, but also peaked between EGCG and ECG, which are the two main active ingredients in TP and far from each other in chromatogram, and thus became an ideal IS, conferring higher accuracy and precision upon the HPLC assay. The validation showed that the HPLC assay described in the present paper completely meets the requirements for excretion study of four catechins in TP.

It is evident that urine and bile must be diluted beforehand with distilled water, otherwise, the interferences from matrix cannot be obviated; A 5-fold dilution is preferred.

The dose of 100 mg/kg was chosen because it is an effective pharmacological dose (Wang *et al*, 2006; Lü *et al*, 2007a) and the middle dose in the plasma pharmacokinetic study in rats (Fu *et al*, 2009). During preliminary experiment ig administration was tried, but the poor oral bioavailability of TP made HPLC-UV assay unable to sensitively detect the four catechins in urine, bile, and feces; In view of this fact, TP was dosed by iv but not ig route.

To date, no reports are available in literature to fully describe the excretion profile of TP, a multi-constituent extract from green tea, but our study did so and has demonstrated that the principal excretory products present as parent compounds in urine, bile, and feces are EC, ECG, and EGC, respectively, by reference to their A_e and f_e. Based on the $f_{e,\ total}$, the excretion of the four catechins in rats is ranked in order of EGC (18.32%) > EC (10.78%) > ECG (2.00%) > EGCG (1.64%), interestingly, just as opposed to the order of their percent content [EGCG (46.81%) > ECG (16.54%) > EC (10.60%) > EGC (6.64%)] in TP; for this opposite relationship one possible explanation is that the ester type catechins EGCG and ECG are partially converted *via* hydrolysis *in vivo* to their corresponding non-ester type catechins EGC and EC, respectively, leading to increased and, consequently, higher $f_{e,\ total}$ of EGC and EC than EGCG and ECG, and also this conversion from EGCG to EGC outweighs conversion from ECG to EC, thus finally resulting in the excretion in order of EGC > EC > ECG > EGCG. Although this explanation remains to be substantiated, our finding is supported by the study of Lee *et al* (Lee *et al*, 1995), who found that EGCG level in plasma was lower than EGC and was not detected in urine in human subjects given 1.2 g TP, and speculated that ester bonds of EGCG and ECG were cleaved by esterase to form EGC and EC.

It is worthy of note that after iv administration of TP the A_e and f_e of EGC in feces greatly exceed that in bile ($P < 0.01$), implying that intestinal active secretion and, therefore, related transporter may be involved in fecal excretion of EGC. Why only EGC among four catechins behaves so is unclear; it may be a possible reflection of the substrate specificity of efflux transporter.

The findings that only insignificant amount of the four catechins are recovered as unchanged compounds

in urine, bile, and feces indicate an extensive metabolic conversion of catechins in the rat body; The presence of multiple hydroxyl groups in their molecules gives us the reason to deduce that the four catechins are mainly excreted as conjugates, most likely glucuronides and sulfates, which are not extracted by EtOAc, an extraction solvent employed in the present study.

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