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Journal Name

ARTICLE

Simultaneous determination of seven catechins in rat plasma by ultra-high performance liquid chromatography tandem mass spectrometry and its application to a pharmacokinetics study

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A rapid, sensitive and selective ultra-high performance liquid chromatography tandem mass spectrometry was developed and validated for the determination and pharmacokinetic investigation of seven catechins in rat plasma. The rat plasma was extracted with simple liquid-liquid extraction using ethyl acetate. Plasma sample was separated by UHPLC on a Hypersil GOLD C₁₈ column (1.9 μm, 50 × 2.1 mm) using a mobile phase consisting of methanol-0.05% formic acid in water with gradient elution. The total run time was 10.5 min and seven catechins were efficiently separated. The detection was performed on a selected reaction monitoring using the respective transitions *m/z* 289.070 → 109.010 for (-)-epicatechin/(+)-catechin, 305.089 → 125.228 for (-)-epigallocatechin/(-)-gallocatechin and 441.150 → 169.325 for (-)-epicatechin gallate and 456.840 → 169.257 for (-)-epigallocatechin gallate/(-)-gallocatechin gallate. Mean recovery of seven catechins was in the range of 84.92-102.18%. The intra- and inter-day precisions (RSD) of these analytes were all less than 6.17% and 5.84%. This method was successfully applied in the pharmacokinetic study of seven catechins in the plasma of rats after oral administration of 700 mg kg⁻¹ tea polyphenols.

Introduction

Tea, not only serves as one of the three main beverages in the world, but also provides some natural polyphenols for food supplements and drug ingredients^{1,2}. Tea polyphenols possess plentiful of biological activities, such as anti-obesity, anti-cancer, anti-inflammatory and anti-viral, which have been widely reported³⁻⁶. Although (-)-epigallocatechin-3-gallate (EGCG) is the most abundant polyphenol in unfermented tea (green tea), the healthy benefits of tea were usually attributed to multi-components of flavan-3-ols. Most of these pharmacological results were obtained using tea polyphenols as material in animal experiments⁷⁻⁹. Furthermore, it was reported that (-)-epigallocatechin (EGC) had potent biological activities¹⁰⁻¹². (-)-Gallocatechin gallate

(GCG) and EGCG are a pair of isomers, but it has been indicated that GCG had similar protective effects on postischemic myocardial dysfunction as EGCG¹³. Other reports also suggested that the GCG had similar activities as EGCG, even better effects^{14,15}. Moreover, (-)-catechin gallate (CG) and GCG were more effective to precipitate cholesterol than (-)-epicatechin gallate (ECG) and EGCG¹⁶. To sum up, except for EGCG, other catechins including EGC, (-)-gallocatechin (GC), GCG and (+)-catechin (C) also contribute to the healthcare function of tea, but were less concerned.

There have been many analytical methods for the determination of tea polyphenols, such as HPLC and LC-MS/MS¹⁷⁻¹⁹. Some reports concerned the determination of catechins in the bio-samples of human and animals²⁰⁻²². Until now, the most reported analytical methods for pharmacokinetics of catechins in bio-samples were mainly for EGCG, ECG and EC^{20,23,24}. The pharmacokinetics of EGC, GC, C and GCG were less reported. Furthermore, restricted by the sensitivity of regular chromatography techniques, the simultaneous quantitative determination of these catechins was usually applied on tea plants rather than bio-samples.

It was reported that bioavailability of tea polyphenols was very low *in vivo*, so ultra-performance liquid

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1 chromatography coupled with tandem mass spectrometry
2 (UHPLC–MS/MS) would be an sensitive tool for the
3 simultaneous determination of these compounds in bio-
4 samples^{25–27}. Therefore, it is worthwhile to develop a
5 simple, precise and accurate UHPLC–MS/MS method for
6 the determination of seven catechins in rat plasma. In this
7 study, the bio-analytical method for the determination of
8 seven catechins as well as their pharmacokinetics in
9 biological fluids was developed. The analytical time and
10 sensitivity could also meet the requirements of high-
11 throughput bio-analysis. Once developed and validated,
12 this method was successfully applied to a pharmacokinetic
13 study in rats.
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16 Experimental

17 Chemicals and reagents

18 EGCG, C, EC, ECG, EGC, GCG, GC were purchased from
19 National Institute for the Control of Pharmaceutical and
20 Biological Products (Beijing, China). Rutin (using as internal
21 standard, I.S.) was purchased from Sichuan Weikeqi
22 Biotechnology Co., Ltd. The purities of above-mentioned
23 ingredients were more than 98% according to HPLC
24 analysis. Methanol and acetonitrile (HPLC-grade) was
25 purchased from Merck (Darmstadt, Germany). HPLC-grade
26 formic acid was obtained from ROE Scientific Inc (Newark,
27 USA). The standardized tea polyphenols extract product
28 was provided by Hangzhou Yibei Co., Ltd. (Zhejiang,
29 China). It contains seven kinds of catechins (54.91% of
30 EGCG, 20.04% of EGC, 11.17% of ECG, 3.37% of EC, 2.13% of
31 GCG, 1.98% GC and 0.51% of C) by the UHPLC-MS
32 determination.
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35 Animal treatment

36 Six male Sprague – Dawley rats (220–250 g) were purchased
37 from the Nanjing Qinglong Experiment Animal Co., Ltd.
38 (Jiangsu province, China). These animals were maintained
39 on a 12 h light-dark cycle (light on from 8:00 to 20:00) at
40 ambient temperature (22–24 °C) and 60% relative humidity.
41 All animal experiments were strictly in accordance with the
42 related guides and ethics regulations and approved by the
43 Institutional Animal Care and Use Committee of Nanjing
44 University of Chinese Medicine. All animals had free access
45 to food and water. The food was removed 12 h before the
46 collection of blood samples. Rats were orally administered
47 tea polyphenols at the dose of 700 mg kg⁻¹. After oral
48 administration, blood samples (0.3 mL) were collected into
49 heparinized tubes *via* the orbital sinus at 5, 10, 20, 40, 60,
50 90, 120, 240, 360, 480, and 720 min after the oral
51 administration. The blood samples were centrifuged at
52 6000 rpm for 5 min.
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Sample preparation

An aliquot of 100 µL plasma was transferred into an
Eppendorff tube containing 20 µL each of 600 ng mL⁻¹ I.S.
and 20% vitamin C solution and then vortex-mixed for 1
min. The sample each extracted once with 1 mL of ethyl
acetate by 3 min of vortex-mixing and then centrifuged at
13000 rpm for 10 min at 4 °C. The upper organic phase was
transferred into another tube and evaporated to dryness by
an Integrated SpeedVac concentrator system (Thermo
Scientific, USA). The residue was dissolved in 100 µL of 20%
acetonitrile aqueous solution and vortex-mixed for 1 min.
After centrifuging at 17000 rpm for 10 min at 4 °C, 5 µL of
the supernatant was injected into UHPLC-MS/MS system
for analysis.

Instrument and analytical conditions

The quantitation of plasma samples was carried out using a
TSQ Vantage UHPLC-MS/MS (Thermo Fisher Scientific, USA)
including the UltiMate 3000 UHPLC, auto-sampler, column
compartment and TSQ mass spectrometer. Separation was
achieved using a Hypersil GOLD column (particle size 1.9
µm; column size 50×2.1 mm; Thermo Scientific, USA)
with a guard column (particle size 3 µm; column size 10×
2.1 mm; Thermo Scientific, USA). The column temperature
was maintained at 35.0±1.0 °C. The mobile phase was
composed of 0.05% formic acid-water (A) and methanol (B)
with the flow rate of 0.30 mL min⁻¹. The linear gradient
condition of mobile phase was 0 – 1.0 min, 10%B; 1.0 – 7.0
min, 10 – 30%B; 7.0 – 7.5 min, 30–70%B; 7.5–8.0min,
70%B; 8.0–8.5 min, 70–10% B; 8.5 – 10.5 min, 10% B. The
API source was operated in the heated electrospray
ionization (H-ESI) mode. During the analyses, the H-ESI
parameters were set as follows: spray voltage, 3000 V for
the negative ion polarity mode; vaporizer temperature, 450
°C; sheath gas pressure, 45 psi; aux gas pressure, 25 psi;
capillary temperature, 350 °C. The collision energies (CE)
were 27, 21, 24, 23 and 38 V for EC/C, EGCG/GCG, EGC/GC,
ECG and rutin (I.S.), respectively.

Selected reaction monitoring (SRM) mode was
employed to detect the target compounds by selected
product ions from the parent ions (EC/C, 289.070 →
109.010; EGCG/GCG, 456.840 → 169.257; EGC/GC, 305.089
→ 125.228; ECG, 441.150 → 169.325; IS, 609.000 →
299.902). The UHPLC-MS/MS data were acquired and
processed by Xcalibur software (version 2.2; Thermo
Scientific, USA).

The mobile phase of UHPLC was screened using
methanol and acetonitrile as organic phase. The mobile
phase consisting of methanol and 0.05% formic acid water
can achieve a better resolution and symmetry of seven
catechins in the chromatogram. Internal standard was
screened using vanilline, quercetin and rutin. In the
negative mode of mass spectrometry, the rutin showed the
best stability and repeatability.

Preparation of standards and quality control (QC) samples

Seven separate primary stock solutions of catechins were prepared in methanol at a concentration of 1 mg mL⁻¹. These stock solutions were then mixed together and continuously diluted with methanol to produce a series of standard or QC working solutions at the desired concentrations. The calibration standards were prepared by adding 20 µL of the standard working solutions into 100 µL of pooled plasma, and then adding 20 µL of 600 ng mL⁻¹ I.S. and 20% vitamin C solution. The final concentrations at 1, 5, 10, 50, 200, 5000 and 10000 ng mL⁻¹ were obtained. The preparation method of standards solution was same as plasma sample preparation. The calibration curves for each catechin were established using different concentrations of catechins according to the pre-determined plasma concentration of catechin of rats orally administrated with tea polyphenols. Low, medium and high levels of QC samples were prepared at 50, 1000 and 5000 ng mL⁻¹ for EGCG, 10, 200 and 1000 ng mL⁻¹ for EGC, ECG and EC, 5, 50 and 200 for GCG, GC and C. All solutions described above were stored at 4 °C.

Method validation

The method was validated for linearity, selectivity, precision, accuracy, matrix effect, extraction recovery and stability according to the guidelines for the validation of bio-analytical methods.

The calibration curves for seven catechins were constructed by plotting peak area ratios of the analytes to I.S. against plasma concentrations using a 1/C² weighted linear least-squares regression model. The linearity of seven catechins determined in spiked rat plasma was obtained using calibration standards in independent runs. The lower limit of quantification (LLOQ) was defined as the lowest concentration with a signal-to-noise ratio of at least 5-fold, and the lower limit of detection (LLOD) was the concentration giving a signal-to-noise ratio at least 2-fold with acceptable accuracy within 20% deviation and precision between 80% and 120%.

The selectivity was evaluated by comparing the chromatograms of six different batches of normal blank plasma obtained from six subjects with those of corresponding standard plasma samples spiked with seven target compounds, I.S. and plasma samples obtained after oral administration of tea polyphenols.

Three concentrations (high, medium and low) of seven standard stock solutions were added to plasma to obtain control samples respectively, and were determined in five separate runs on the same day for intra-day and on three consecutive days for the inter-day accuracy variation. The accuracy of the method was determined by calculating the percentage deviation observed during the analysis of quality controls and expressed as the relative error (RE).

$$RE\% = \frac{(\text{measured value} - \text{theoretical value})}{\text{theoretical value}} \times 100.$$

The matrix effects were measured by comparing the peak areas of the analytes dissolved in the pre-treated blank plasma with that of pure standard solution containing equivalent amounts of the analytes. The extraction recovery was calculated by comparing the peak areas obtained from extracted spiked samples with those of unextracted spiked samples at corresponding concentrations. The extraction recovery and matrix effect of the I.S. were also evaluated using the same procedure.

The stability of the analytes in rat plasma was assessed by analysing QC samples at three concentration levels approached to three different conditions. The short-term stability was determined with untreated QC samples stored for four hours at room temperature. The freeze-thaw stability was determined after three freeze-thaw cycles (-20 °C to room temperature as one cycle). The long-term stability was determined with untreated QC samples stored for 30 days at -20 °C.

Pharmacokinetic study

The dosing solution of tea polyphenols (100 mg mL⁻¹) was prepared by suspending the required amounts in water. The same dose of tea polyphenols at 700 mg kg⁻¹ was given to each rat. Approximately, 0.3 mL of blood samples were collected from the vein of the eye ground at 5, 10, 20, 40, 60, 90, 120, 240, 360, 480, and 720 min after oral administration. The blood samples were immediately heparinized and centrifuged at 6000 rpm for 5 min. Supernatant fluids were divided into 0.2 mL aliquots and stored in 1.5 mL polypropylene tubes at -20 °C prior to analysis.

Data analysis

The maximum concentration (C_{max}) of catechins in plasma and the time to reach the maximum concentration (T_{max}) were directly obtained from the observed values. Other pharmacokinetics parameters, such as area under concentration-time curve (AUC) and mean residence time (MRT), terminal elimination half-life ($T_{1/2}$) and clearance (CL) were calculated using Drug and Statistics 3.0 (DAS 3.0, Mathematical Pharmacology Professional Committee of China, Shanghai, China).

Results and discussion

Mass spectrometry

In the full scan mass spectra, the deprotonated molecular ions [M-H]⁻ of (1,2) C/EC m/z at 289.070, (3,4) EGCG/GCG m/z at 456.840, (5) ECG m/z at 441.150 (6,7) EGC/GC m/z at

305.089, and (8) I.S. m/z at 609.000 were stable and exhibited higher abundance. Under the product ion scan mode, the intensive product ions were m/z 109.010 and 187.017 from m/z 289.070 (1,2), m/z 169.257 and 304.788 from m/z 456.840 (3,4), m/z 169.325 and 289.32 from m/z 441.150 (5), m/z 125.228 and 219.044 from m/z 305.089 (6,7), and 299.902 from m/z 609.000 (8). The mass spectrometric parameters were optimized to obtain the higher signal for both precursor ions and product ions as shown in Fig. 1.

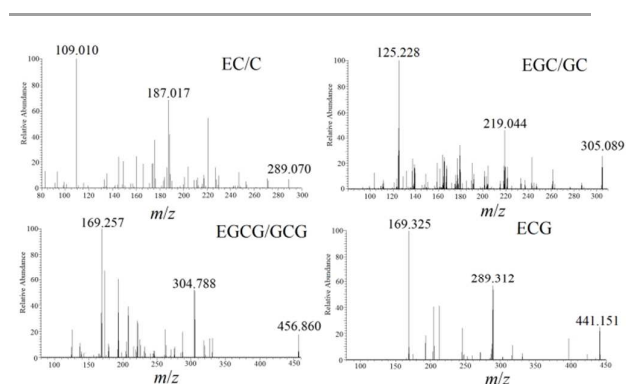


Fig. 1. The parent ions and product ions of mass spectra (MS/MS) of EC/C, EGC/GC, EGCG/GCG and ECG

Method validation

Linearity

Table 1 lists the linearity parameters, LLOQ and LLOD of the seven analytes. The correlation coefficients of these calibration curves were all higher than 0.995.

Table 1 Calibration curve, and linear range of the seven catechins

Analytes	Calibration curve	r	Linear range (ng mL ⁻¹)	LOQ (ng)	LOD (ng)
EC	$Y=0.00115X+0.00135$	0.9991	5-5000	5	1
EGC	$Y=0.00081X-0.00039$	0.9994	5-5000	5	1
ECG	$Y=0.00539X+0.01339$	0.9997	5-5000	5	1
EGCG	$Y=0.00243X+0.00774$	0.9996	10-10000	10	5
C	$Y=0.00081X+0.00058$	0.9991	1-500	1	0.5
GC	$Y=0.00134X+0.00145$	0.9994	1-500	1	0.5
GCG	$Y=0.00281X+0.00623$	0.9992	1-500	1	0.5

Selectivity

The selectivity of the method towards endogenous plasma matrix was evaluated in six rat plasma. Blank plasma yielded relative clean chromatograms without interfering

peaks both to the analytes or I.S.. The retention times of C, EC, ECG, EGC, EGCG, GCG, GC and I.S. were 3.28, 5.16, 7.09, 2.89, 4.86, 6.16, 1.16 and 8.88 min, respectively. Using the main product ions as referenced standard, these analytes in plasma samples were confirmed by the product ions 169.187/304.928 and 169.467/304.98 for EGCG/GCG; 109.126/187.053 and 109.196/187.333 for EC/C; 125.018/219.044 and 125.018/218.974 for EGC and GC; 168.975 and 289.102 for ECG at corresponding retention time. All the peaks of the analytes and I.S. in plasma samples were unambiguously identified by comparison of retention time, parent and product ions with reference standards as shown in Fig. 2.

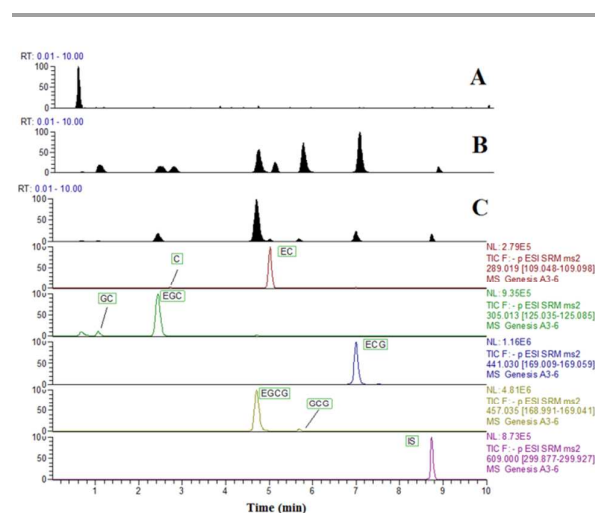


Fig. 2. Representative UHPLC-MS chromatograms of (A) blank plasma sample, (B) catechins standards and I.S., and (C) a 2 h plasma sample from an oral (700 mg kg⁻¹) dosed animal

Precision and Accuracy

The result (ESI Table 1†) showed that the intra- and inter-day precisions and accuracies of seven catechins at three concentration levels (low, medium and high) (RSD) of these analytes were all less than 6.17% and 5.84%.

Recovery and matrix effect

The extraction recoveries (absolute recovery) of the examined compounds from rat plasma samples were determined at different concentrations. These samples were subjected to the methanol extraction procedure and injected into the UHPLC-MS system. The extraction recoveries of analyzed compounds from spiked rat plasma were evaluated at the low, medium and high concentrations. The recoveries in plasma were obtained for C (from 88.46% to 91.78%), EC (from 84.92% to 93.95%), ECG (from 86.79% to 92.19%), EGC (from 85.40% to 93.66%), EGCG (from 91.06% to 102.18%), GCG (from 85.82% to 92.53%), and GC (from 92.90% to 95.55%). To study the effect of the matrix on analytes quantification

with respect to consistency in signal suppression, the matrix effect was checked in three different batches of heparinized plasma. Three replicates for each of these three concentrations were prepared from different batches of plasma. It showed that the analytes in this study exhibited no matrix effect (ESI Table 2†).

Stability

The results (ESI Table 3†) showed that the analytes in rat plasma were all stable for 4 h at room temperature, three cycles of freeze-thaw, 30 d at -20 °C.

Pharmacokinetics of seven catechins

The plasma concentrations of seven catechins for the rats treated with tea polyphenols (700 mg kg⁻¹) were plotted against time as shown in Fig. 3. The pharmacokinetics of EGC, GCG, GC and C in rat plasma was reported in the present study. The plasma concentration-time data of EGC, GCG, GC and C were fitted in a one-compartment oral input model. The mean estimated pharmacokinetic parameters for seven catechins were listed in Table 2. The observed T_{max} 's were 20, 20, 20 and 10 min, and the C_{max} 's were 2418.08, 354.90, 214.09 and 61.62 ng mL⁻¹ for EGC, GCG, GC and C, respectively. The $MRT_{(0-t)}$ were 221.17, 322.58, 172.95 and 46.59 min for EGC, GCG, GC and C, respectively. The absorption rate constants (K_a) of EGC, GCG, GC and C were 0.005, 0.006, 0.008 and 0.041. A bimodal phenomenon of seven catechins was presented in Fig. 3, which were in parallel with the literatures and was probably due to distribution, re-absorption and enterohepatic circulation.

In the previous paper²⁸, when rats were orally given 100 mg kg⁻¹ EGCG, the C_{max} was determined as 1.52 µg mL⁻¹. In the present study, the oral administrated EGCG dose was about 385 mg kg⁻¹. The C_{max} of plasma EGCG was 4.92 µg mL⁻¹, which was correspondingly increased with the dosing increased. The T_{max} 's of plasma EGCG in two studies were similar, that was 20 and 24 min respectively. The $T_{1/2}$'s showed a significant difference, when the given dose of EGCG was highly increased, the $T_{1/2}$ value was also increased. In the present study, the $T_{1/2}$ was 399.72±156.97 min, which was higher than that of $T_{1/2}$ (48±13 min) obtained from the single EGCG administration. The interaction of catechins in tea polyphenols may affect the pharmacokinetics of individual catechin *in vivo*, so that decreased the metabolism of EGCG.

Furthermore, other studies reported the pharmacokinetics of EGCG, EGC and EC, but mostly human was the subject in these clinical pharmacokinetics studies^{29,30}. In these studies, the T_{max} 's of plasma EGCG, EGC and EC of human were delayed than those of rats. Chen *et al.* studied the pharmacokinetics of EGCG, EGC and EC of rats orally administered decaffeinated green tea extract³¹. It showed that T_{max} 's values of plasma EGCG, EGC and EC were higher than the results observed in the present study. These differences may be caused by counting the conjugated catechins into the plasma catechins, so that the pharmacokinetics parameters represented the integrated catechins' parameters.

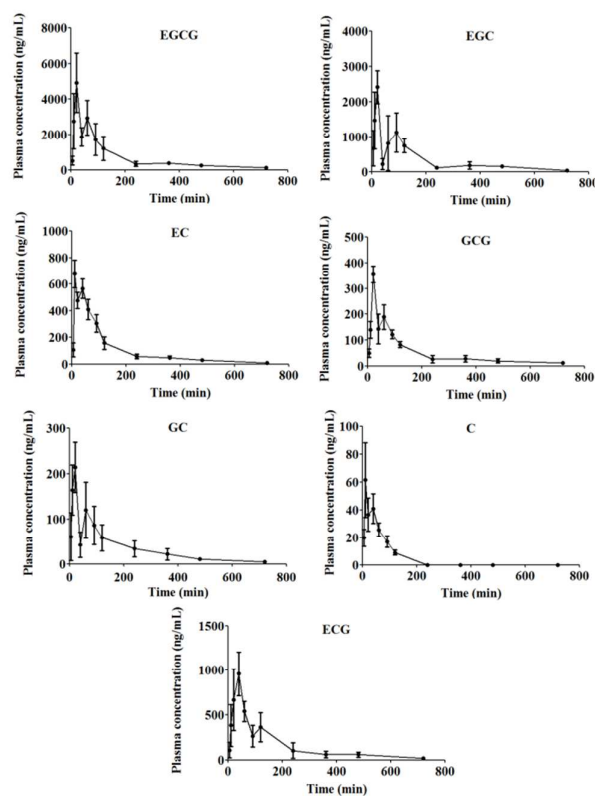


Fig. 3. The profiles of mean plasma concentration–time of seven catechins after oral administration of tea polyphenols (n=6, mean±SD).

Table 2 Pharmacokinetic parameters of seven catechins in male SD rats following oral administration of tea polyphenols (mean±SD, n=6)

Analytes	T_{max} (min)	C_{max} (ng mL ⁻¹)	$T_{1/2}$ (min)	$AUC_{(0-t)}$ (mg min L ⁻¹)	$AUC_{(0-∞)}$ (mg min L ⁻¹)	$MRT_{(0-t)}$ (min)	$MRT_{(0-∞)}$ (min)	CL (mL min ⁻¹ kg ⁻¹)	K_a
EC	10.00±0.00	676.59±104.01	197.74±63.96	75.66±6.31	79.67±8.65	140.09±18.16	183.53±54.69	8.88±1.01	0.011±0.001

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EGC	20.00±0.00	2418.08±468.83	230.73±70.89	248.26±44.35	251.32±44.95	221.17±34.25	240.46±34.51	2.87±0.56	0.005±0.00
ECG	40.00±0.00	958.47±245.86	509.05±17.498	125.10±41.25	132.69±39.81	226.40±13.83	359.41±71.81	5.71±1.79	0.033±0.029
EGCG	20.00±0.00	4927.82±1663.31	399.72±156.97	558.63±153.72	588.93±158.92	253.69±57.401	350.25±107.62	1.28±0.42	0.010±0.009
C	10.00±0.00	61.62±26.895	42.09±11.57	3.27±0.79	3.88±0.67	46.59±3.73	69.15±16.89	185.29±33.78	0.041±0.022
GC	20.00±0.00	214.09±55.26	184.53±46.03	24.90±9.60	26.14±9.50	172.95±17.12	217.60±24.78	29.31±8.64	0.008±0.001
GCG	20.00±0.00	354.90±32.89	450.64±229.42	43.50±8.96	46.70±10.0	322.58±26.14	322.58±26.14	455.26±173.53	0.006±0.002

Conclusions

A UHPLC–MS/MS method for the simultaneous determination of seven catechins in rat plasma was developed and validated. The validated method showed acceptable data for all the validation parameters, with adequate sensitivity and selectivity for their simultaneous quantification in pharmacokinetics study. To the best of our knowledge, this is the first report about pharmacokinetics study of EGC, GCG, GC and C in rat plasma using an UHPLC–MS/MS method.

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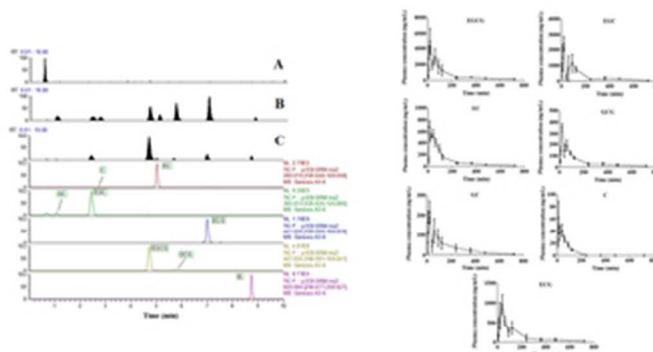
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HPLC-MS/MS for the pharmacokinetics of seven catechins of rats orally administered tea polyphenols

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