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Investigation of intestinal absorption and disposition of green tea catechins by Caco-2 monolayer model

Li Zhang, Ying Zheng, Moses S.S. Chow, Zhong Zuo*

School of Pharmacy, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong

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Abstract

The current study was designed to investigate the absorption mechanism and identify the possible disposition pathways of green tea catechins (GTC), including epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG), during their absorption across small intestine by Caco-2 monolayer model. The transport of each GTC from both apical to basolateral and basolateral to apical directions was measured in the absence and the presence of MK571, an MRP inhibitor. HPLC and LC/MS were employed to identify the possible metabolites of the four GTC formed during their bidirectional transport processes. The results indicated that the four GTC showed limited transepithelial absorption with relatively small P_{app} values. However, significant efflux mediated by MRP was observed during the secretion of GTC, especially the non-gallated catechins. Methylation and sulfation were the main biotransformation pathways of GTC during their secretion transport and the efflux of the related metabolites seem to be mediated by MRP.

Keywords: Green tea catechins; Caco-2 cells; Efflux; Absorption; Metabolism

1. Introduction

Green tea, a popular beverage in many countries, contains catechins (flavan-3-ol) as its major beneficial substances (Takehiko and Mujo, 1997). Among the catechins, epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), and epicatechin (EC) are the major active components in green tea (Fig. 1). A series of pharmacological effects, including anti-carcinogenesis activity, anti-oxidative activity and anti-platelet aggregation activity, had been reported from both animal and human studies of green tea catechins (GTC) (Katiyar and Mukhtar, 1996; Young et al., 2002; Duffy et al., 2001).

Although the beneficial effects of GTC are well recognized, the pharmacokinetics property of GTC is not completely elucidated. Moreover, serious pre-systemic eliminations of GTC had been reported in animals

* Corresponding author. Tel.: +852 2609 6832; fax: +852 2603 5295.
E-mail address: joanzuo@cuhk.edu.hk (Z. Zuo).

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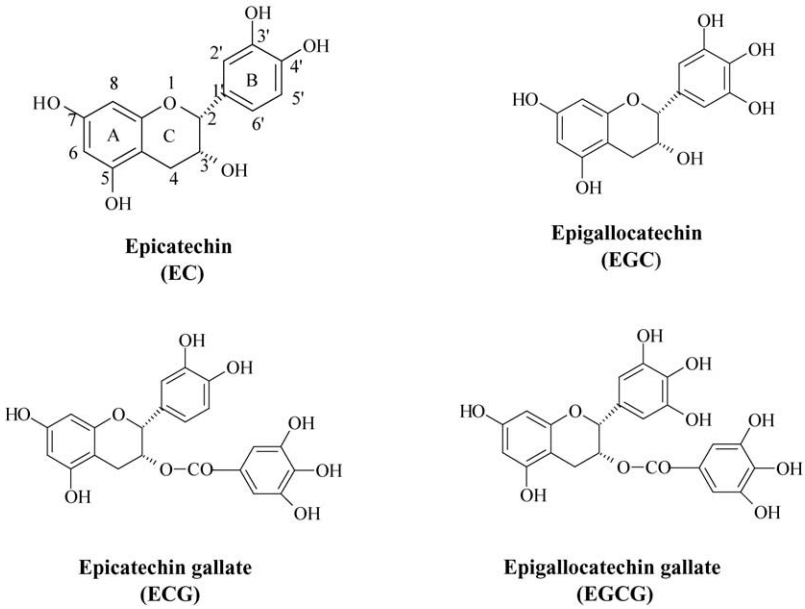

Fig. 1. Structures of green tea catechins.

after oral administration (Chen et al., 1997; Zhu et al., 2000). Due to the unique anatomical location, gut wall may represent an important and highly sensitive site for orally administered drugs. Recently, a few reports on the first pass metabolism of flavonoids demonstrated that extensive phase II metabolism, such as glucuronidation, in gut could contribute to the overall low oral bioavailability (Crespy et al., 1999; Andlauer et al., 2000; Walle et al., 2001). Therefore, it is necessary to investigate whether this is one of the potential factors responsible for the low oral bioavailability of GTC.

Moreover, few studies reported the identification of metabolites of GTC in small intestine even though the metabolites of GTC in urine, bile and plasma were reported (Harada et al., 1999; Okushio et al., 1999; Meng et al., 2001; Kida et al., 2000). One of the reasons is that it is technically difficult to investigate the transport and metabolism of GTC in human small intestine. Recently, the human intestinal Caco-2 cell monolayer model has proven to be the most popular in vitro model to rapidly assess the cellular permeability of potential drug candidates, to elucidate pathways of drug transport as well as to study the pre-systemic drug metabolism in gut (Meunier et al., 1995). Besides, similar to the gut, Caco-2 cells also express transporters, such as multi-drug resistance protein (MRP) that is involved in the excretion of the conjugated metabolites from cells (Hirohashi et al., 2000). It has also been suggested that metabolism/active efflux in the small intestine is involved in the poor absorption of many drugs. The transport and metabolism study of the tea flavonoid (−)-epicatechin (EC) by the Caco-2 model suggested an important role of MRP2 in the bioavailability of EC and possibly other tea flavonoids (Vaidyanathan and Walle, 2001). Therefore, the present study aims to investigate the absorption and metabolism of GTC during their absorption across the small intestine by Caco-2 cell model.

2. Materials and methods

2.1. Materials

(−)-Epicatechin, (−)-epigallocatechin, (−)-epicatechin gallate, (−)-epigallocatechin gallate, lucifer yellow were purchased from Sigma Chemical Co., USA. MK571 was supplied by Biomol Research Laboratories Inc., USA. Isoquercitrin (IQ), used as inter-

nal standard for determining the concentration of EC and EGCG, was from Carl Roth, Germany. HPLC-grade methanol and acetonitrile were from Labscan Asia Co. Ltd., Thailand. Other chemical reagents used were at least of analytical grade. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), 0.05% trypsin–EDTA, penicillin–streptomycin, and non-essential amino acids were obtained from Gibco BRL, Life & Technologies, USA. Phosphate-buffered saline tablets were purchased from Sigma Chemical Co., USA.

2.2. Methods

2.2.1. Cell culture

Caco-2 cells from the American type culture collection (ATCC) were cultured in Dulbecco's modified Eagle's medium at 37 °C, supplemented with 10% fetal bovine serum, 1% non-essential amino acids, in an atmosphere of 5% CO_2 and 90% relative humidity. Cells were subcultured at 80–90% confluence by trypsinization with 0.05% trypsin–EDTA and plated onto six-well plates Transwell® inserts (24 mm i.d., 0.4 μm pore size, 4.71 cm^2, polycarbonate filter, Corning Costar Co., NY) coated with a collagen layer at a density of 3×10^5 cells/well and cultured for 21 days prior to transport experiments. TEER was used to monitor the integrity of the monolayer. Monolayer with TEER above 600 Ω cm^2 (after subtracting the back group value of the transwell) was employed in the present study. Caco-2 cells grown in Transwell® at passage 32–45 were used for the experiment.

2.2.2. Bi-directional transport studies of green tea catechins

The transport buffer employed in the transport studies contained 0.01 M of phosphate buffer saline (PBS^+), which was supplemented with 0.45 M calcium chloride and 0.4 M potassium chloride and adjusted to pH 6.0. Transwell®, with Caco-2 cells grown on them for 21 days, were rinsed twice and equilibrated with PBS^+ transport buffer at 37 °C for 15 min before the transport experiment. In the bi-directional transport study, 50 μM of each GTC in PBS^+ was loaded into the apical (AP) (1.5 ml transport buffer) or basolateral (BL) (2.6 ml of transport buffer) side, the so-called the donor side. Aliquots of 0.5 ml samples were taken from the other side, the so-called receiver side at different time intervals (30, 60, 90, 120, 150, 180 min) during the experiment. Same volume of blank PBS^+ was replaced to the receiver chamber after each sampling. Samples taken from the transport study were then acidified with ascorbic acid solution, containing 1% ascorbic acid and 0.28% H_3PO_4, to reach pH 2.5 (Chen et al., 1998) and stored at −80 °C until analysis. The P_{app} was calculated as described previously (Artursson and Karlsson, 1991; Ingels et al., 2004).

Efflux ratio or secretion ratio was used to evaluate the extent of efflux (Liang et al., 2000; Eagling et al., 1999; Faassen et al., 2003). The calculation was performed as the following equation:

$$\text{efflux ratio} = \frac{P_{\text{app}_{\text{BtoA}}}(\text{mean})}{P_{\text{app}_{\text{AtoB}}}(\text{mean})}$$

where $P_{\text{app}_{\text{BtoA}}}(\text{mean})$ is the average of the permeability coefficient from BL to AP, $P_{\text{app}_{\text{AtoB}}}(\text{mean})$ the average of the permeability coefficient from AP to BL.

2.2.3. Transport study of lucifer yellow

Lucifer yellow was commonly employed as a paracellular marker for determining the integrity of the Caco-2 monolayer. The transport studies from apical side to basalateral side with 0.33 mg/ml of lucifer yellow at the apical chamber was performed as described in Section 2.2.2.

2.2.4. Inhibition of transport

To investigate the influence of the multi-drug resistant associate protein (MRP) on the transport of the selected GTC, 50 μM of MK571 was preloaded at the apical chambers for 20 min. The bi-directional transport studies in the presence of 50 μM MK571 at the donor chambers were then performed following the method described in Section 2.2.2 (Vaidyanathan and Walle, 2001).

2.2.5. HPLC analysis of four green tea catechins

A 175 μl sample was spiked with 25 μl of various internal standards as described in Table 1. ODS reversed-phase column (4.6 mm i.d. × 250 mm, 4.5 μm, Beckman) was chosen for the separation. Electrochemical detector (ECD) with potential set at 1000 mV was used for the detection of GTC. The specific mobile phase used for the analysis of each compound was listed in Table 1.

Table 1
HPLC conditions and internal standards (IS) used for analysis of each catechin

Compounds	Flow rate (ml/min)	Time (min)	*Percentage of each eluent* (%)			IS
			Methanol	ACN	Buffer[a]	
EC	1	0	0	10	90	IQ
		15	80	0	20	
		20	0	10	90	
EGC	1	0	0	5	95	EC
		20	0	15	85	
		25	0	5	95	
EGCG	1	0	0	10	90	IQ
		20	5	25	70	
		22	0	10	90	
ECG	1	Identical mobile phase program as EGCG				EC

[a] 25 mM NaH_2PO_4 (pH 2.5).

2.2.6. Identification of metabolites with LC/MS

For identification of the metabolites of EC, EGCG and ECG, the gradient began with 10% eluent A (acetonitrile) and 90% eluent B (0.04% formic acid), and was changed linearly to 30% eluent A and 70% eluent B in 20 min. Then the gradient was changed back to 10% eluent A and 90% eluent B in 2 min.

The gradient for identification of metabolites of EGC began with 10% methanol and 90% water with 0.04% formic acid (pH 3.0) and was changed linearly to 60% methanol and 40% water in 15 min and remained at this percentage for another 5 min. Then the gradient was changed back to 10% methanol and 90% water in the next 2 min followed by equilibrating for another 3 min.

ODS reversed-phase column (4.6 mm i.d. × 250 mm, 4.5 μm, Beckman) was eluted with gradient at a flow rate of 1 ml/min. Twenty percent of eluent was introduced into mass spectrometer and the other 80% was splitted off.

An API 2000 Triple Quadrupole LC/MS/MS spectrometer equipped with two Perkin-Elmer PE-200 series micro-pumps and auto-sampler (Perkin-Elmer, Norwalk, CT, USA) were used to perform the analysis. Negative mode was set for the analysis. Other working mass spectrometer parameters were: orifice voltage, −82 V; ring voltage, −230 V; nebulization gas, 23 psi; auxiliary gas, 40 psi; nebulizer temperature, 400 °C. Possible metabolites of each GTC, including methylated conjugate, sulfate conjugate, glucuronidated conjugate, methylated sulfate conjugate, methylated glucuronidated conjugate, were monitored by targeting at their related deprotonated molecular ions.

Table 2
P_{app} values of the four catechins with and without the treatment of MK571 ($n = 3$)

	Transport in the absence of MK571			*Transport in the presence of MK571*		
	$P_{app} \pm$ S.D. ($\times 10^{-7}$ cm/s)		Efflux ratio	$P_{app} \pm$ S.D. ($\times 10^{-7}$ cm/s)		Efflux ratio
	AP to BL	BL to AP		AP to BL	BL to AP	
EC	1.39 ± 0.082	29.96 ± 1.24	21.55	3.50 ± 0.22[a]	5.25 ± 0.39[b]	1.50
EGC	1.49 ± 0.13	7.72 ± 0.43	5.18	3.31 ± 0.21[a]	3.33 ± 0.21[b]	1.01
ECG	0.96 ± 0.15	3.86 ± 0.73	4.02	N/A	N/A	N/A
EGCG	0.83 ± 0.24	1.52 ± 0.15	1.83	N/A	N/A	N/A

N/A: not applicable.
[a] P_{app} values (AP to BL) in the absence of MK571 are significantly different from that (AP to BL) in the presence of MK571 ($P < 0.05$).
[b] P_{app} values (BL to AP) in the absence of MK571 are significantly different from that (BL to AP) in the presence of MK571 ($P < 0.05$).

2.2.7. Data analysis

Reported values represent mean ± S.D. ($n = 3$). Statistical significant difference was evaluated by Student's t-test with a significant level of $P < 0.05$.

3. Results

3.1. Bidirectional transport of four green tea catechins

When four GTC were loaded to the apical chambers, no significant transepithelial transport of GTC was observed. As shown in Table 2, the P_{app} values for the absorption transport of GTC are similar to that of lucifer yellow ($1.63 \pm 0.12 \times 10^{-7}$ cm/s). Due to the P_{app} values which are much less than 1×10^{-6} cm/s from the absorption direction of GTC, it is indicated that permeability of GTC through lipid bilayer were not favorable, which may be associated with their low oral bioavailability of less than 20% (Yee, 1997). In secretion transport, all studied GTC exhibited efflux with non-gallated catechins (EC and EGC) showing more extensive efflux than the gallated catechins (EGCG and ECG). The efflux of EC and EGC were almost completely inhibited by MRP inhibitor (MK571), which indicated that MRP might play an important role on the efflux of GTC.

3.2. Metabolites of EC

As shown in Fig. 2A, in the BL to AP transport experiment, several metabolites (M1, M2, M3) of EC were observed in the apical chambers as early as 30 min and their amount accumulated for the following 2.5 h. However, none of the above metabolites was found in the receiver chamber during the whole process of the AP to BL transport experiment (Fig. 2B).

The deprotonated molecular ions of EC (*m/z*, 298) and its possible metabolites (EC sulfate conjugate (*m/z*, 369), methylated EC (*m/z*, 303), methylated EC sulfate conjugate (*m/z*, 383), EC glucuronidated conjugate (*m/z*, 465) and methylated EC glucuronidated conjugate (*m/z*, 479)) were targeted by mass spectrometry with electrospray ionisation in a negative mode. The results indicated that the metabolites of EC were mainly sulfate conjugate, methylated EC, and methylated sulfate conjugate of EC (Fig. 3A). Among them, EC sulfate conjugate was the major one. Its full scanning shown in Fig. 4 indicated the molecular ion of EC sulfate conjugate was at *m/z* 369. The predominant product ion of EC sulfate conjugate was its deprotonated aglycone, EC, at *m/z* 289. Based on the lipophilicities of the above metabolites, it was speculated that M1, M2, M3 could be EC-sulfate, Me-EC-sulfate, Me-EC, respectively.

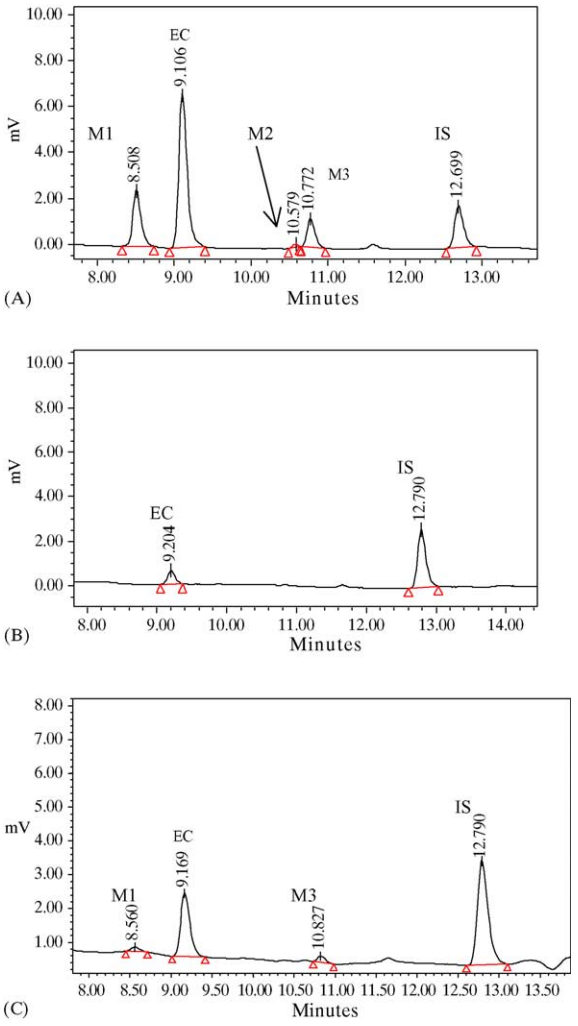

Fig. 2. HPLC/ECD chromatograms of the samples taken from receiver sides at the end of transport experiment. (A) Basal loading and apical sampling. (B) Apical loading and basal sampling. (C) Basal loading and apical sampling in the presence of MK571. M1, M2, M3: metabolites of EC.

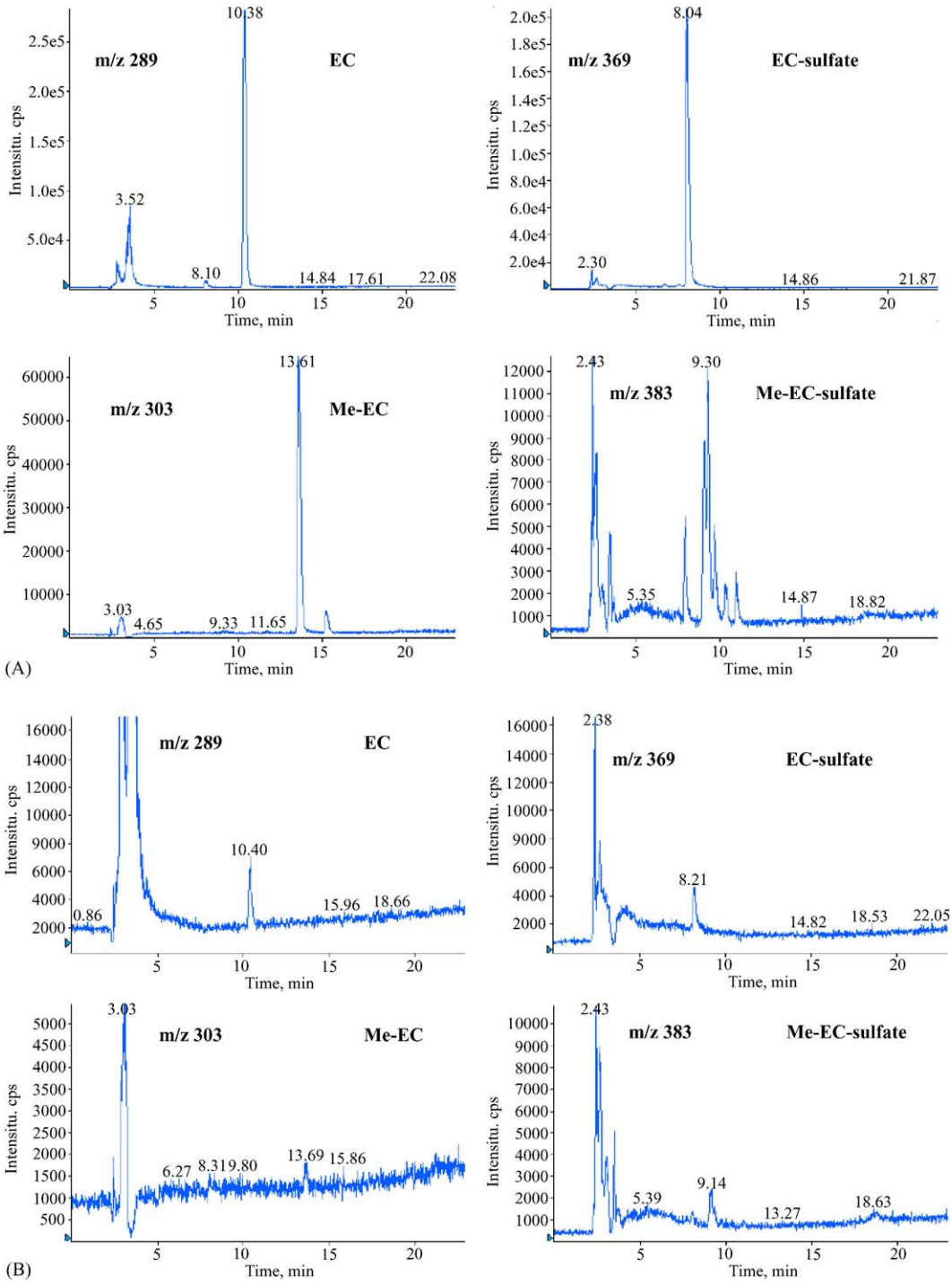

Fig. 3. LC/MS chromatograms of the samples taken from receiver sides at the end of the transport experiment of EC. (A) Basal loading and apical sampling. (B) Apical loading and basalateral sampling. EC: epicatechin, EC-sulfate: epicatechin sulfate conjugate, Me-EC: methylated epicatechin, Me-EC-sulfate: methylated epicatechin sulfate conjugate.

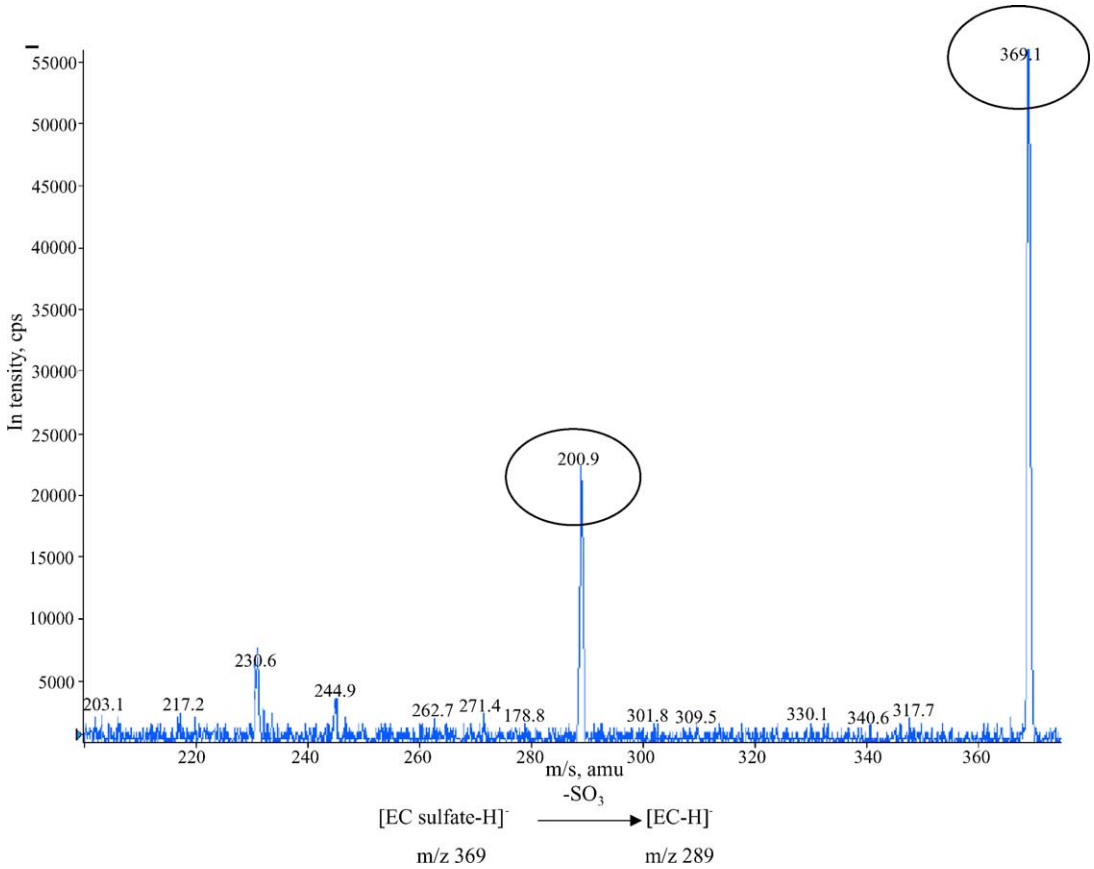

Fig. 4. Mass spectrum of EC sulfate conjugate.

The samples in receiver chambers of the AP to BL transport also showed the same types of metabolites formed as that in the apical samples from the BL to AP transport (Fig. 3B), but with much fewer amount than the later one.

3.3. Metabolites of EGC

HPLC/MS analysis using selective ion monitoring mode (SIM) targeted at the deprotonated molecular ion of EGC (*m/z*, 305) and possible metabolites of EGC, which include sulfate conjugate of EGC (*m/z*, 385), methylated EGC (*m/z*, 319), methylated sulfate conjugate of EGC (*m/z*, 399), EGC glucuronidated conjugate (*m/z*, 481) and methylated EGC glucuronidated conjugate (*m/z*, 495). The results confirmed the occurrence of sulfate conjugate of EGC, methylated EGC and methylated sulfate conjugate of EGC in the receiver chambers during secretion (Fig. 5). In addition, none of the above metabolites was found in the receiver chamber during absorption transport (AP to BL).

3.4. Metabolites of EGCG and ECG

Several metabolites were found at the apical chambers during secretion transport of EGCG and ECG. Identification of the metabolites with HPLC/MS using SIM showed that there were methylated EGCG (*m/z*, 471), sulfate conjugate of EGCG (*m/z*, 537) and methylated sulfate conjugate of EGCG (*m/z*, 551) formed in the apical side after loading the EGCG in the basalateral side (Fig. 6A).

For ECG, there were marginal amount of methylated ECG (*m/z*, 455), sulfate conjugate of ECG (*m/z*, 521) and methylated sulfate conjugate of ECG

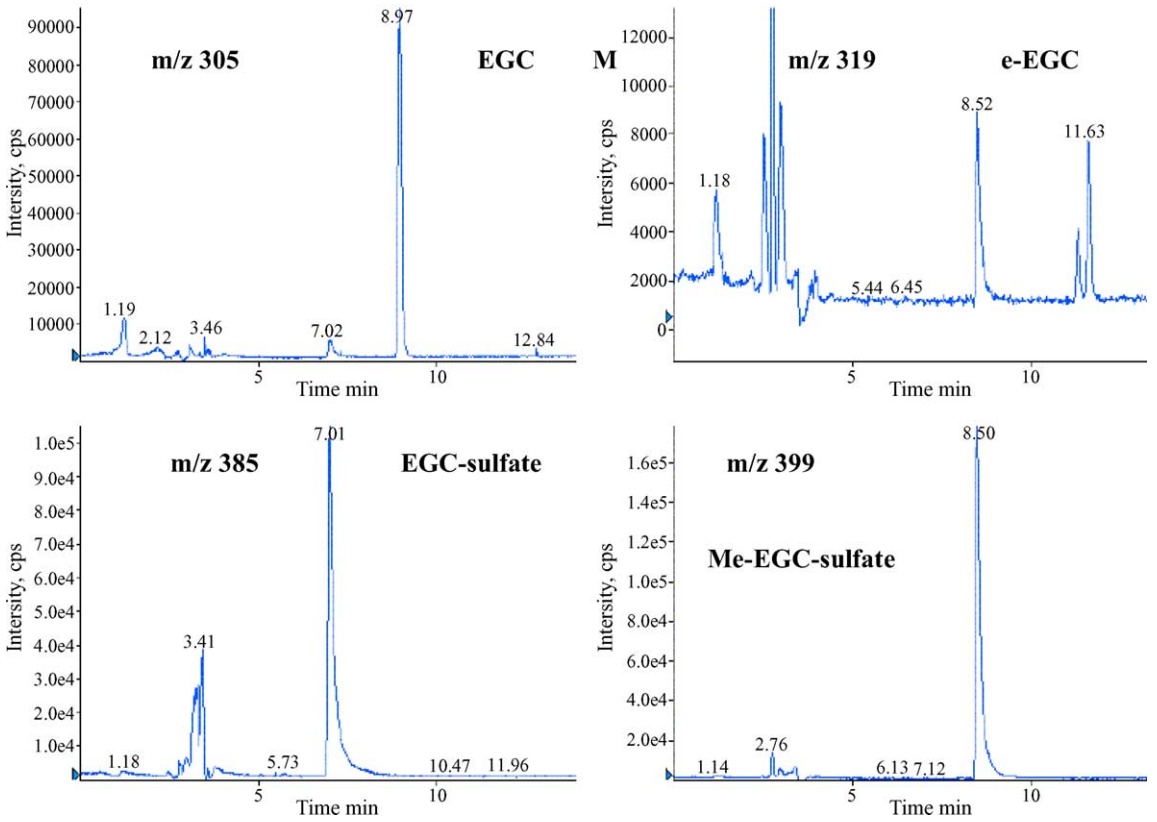

Fig. 5. LC/MS chromatograms of the samples taken from receiver sides at the end of transport experiment of EGC (basal loading and apical sampling). EGC: epigallocatechin, EGC-sulfate: epigallocatechin sulfate conjugate, Me-EGC: methylated epigallocatechin, Me-EGC-sulfate: methylated epigallocatechin sulfate conjugate.

(*m/z*, 535) formed in receiver chambers during secretion transport of ECG (Fig. 6B). Similarly, none of the above metabolites was observed at the receiver chambers in absorption transport of both EGCG and ECG.

3.5. Inhibition transport of metabolites of EC and EGC

As demonstrated in Fig. 2C, the presence of MK571 substantially inhibited the transportation of all the identified metabolites of EC suggesting that the metabolites of EC might be the substrates of MRP. Similarly, transport of metabolites of EGC was also inhibited by MK571 (Fig. 7), indicating that the metabolites of EGC might be the substrate of MRP as well.

4. Discussion

Several metabolites of GTC generated by Caco-2 cell were identified in the transport experiment. They were mainly sulfated and methylated metabolites. Methylation tends to take place at the 3′-OH or 4′-OH position of EC (Kuhnle et al., 2000; Baba et al., 2001). Sulfate conjugate seems to be the major metabolites of EC in human (Vaidyanathan and Walle, 2002), while glucuronide was the major metabolite of EC in rat (Vaidyanathan and Walle, 2002; Kuhnle et al., 2000). Since Caco-2 cell is originated from human, types of the metabolites formed from the present study were consistent with the previous findings from human with no glucuronidated metabolites observed for any of the studied GTC.

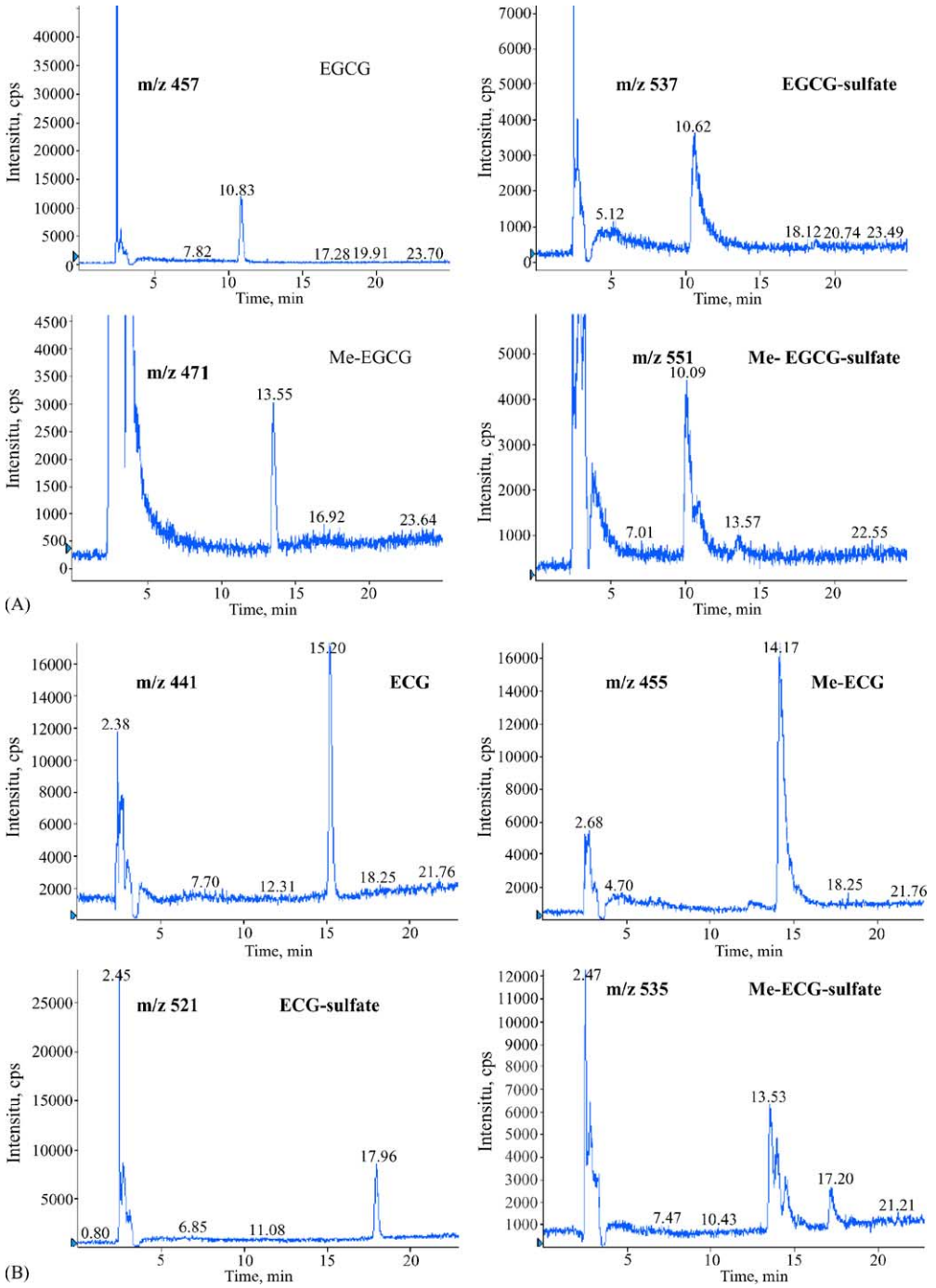

Fig. 6. LC/MS chromatograms of the samples taken from the receiver sides at the end of transport experiment of EGCG (A) and ECG (B) (basal loading and apical sampling). EGCG: epigallocatechin gallate, EGCG-sulfate: epigallocatecin gallate sulfate conjugate, Me-EGCG: methylated epigallocatechin gallate, Me-EGCG-sulfate: methylated epigallocatechin gallate sulfate conjugate, ECG: epicatechin gallate, ECG-sulfate: epicatechin gallate sulfate conjugate, Me-ECG: methylated epicatechin gallate, Me-ECG-sulfate: methylated epicatechin gallate sulfate conjugate.

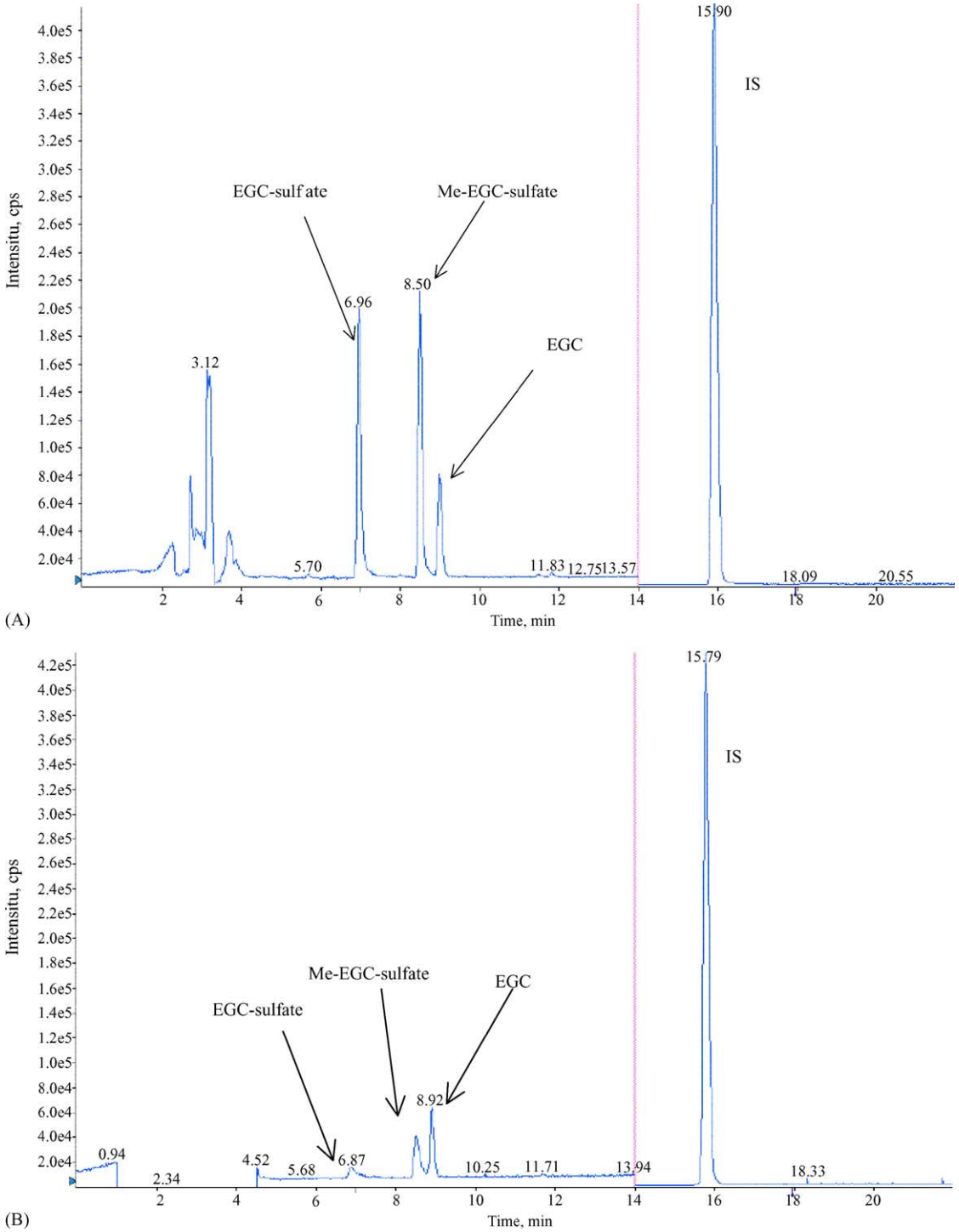

Fig. 7. LC/MS chromatograms of the samples taken from receiver sides at the end of transport experiment of EGC. (A) Basal loading and apical sampling. (B) Basal loading and apical sampling in the presence of MK571.

It has been reported that the metabolites of EGC are 4′-*o*-methyl EGC, methyl-EGC-*o*-sulfate and EGC sulfate conjugate in humans (Meng et al., 2001; Li et al., 2001). Consistently, the present study also found the same type of major metabolites of EGC, i.e. EGC sulfate conjugate, methylated EGC and methylated EGC sulfate conjugate formed in Caco-2 cell model.

Methylation of EGCG at 4′-OH and 4″-OH were previously reported (Okushio et al., 1999; Lu et al., 2003; Kida et al., 2000). The methyl group was also found to conjugate at the 4″-OH of ECG (Kida et al., 2000). As to the type of metabolites of EGCG and ECG, our results are also consistent with previous finding.

For Caco-2 cell derived from human colon carcinoma, expression of human phase I and phase II metabolizing enzymes has been verified in this cell line (Sun et al., 2002). Therefore, the metabolites found in the present study indicated that similar types of metabolism of GTC might also be found in human small intestine. However, Sun et al. also found that the extent of expression of metabolizing enzymes in the Caco-2 cell was lower than that in vivo. In terms of the extent of the metabolism of GTC in the intestine, the present results may not completely apply to the in vivo situation quantitatively.

It is also interesting that the metabolites of GTC preferentially appeared in the apical chambers during secretion while only a little of those metabolite were found in the basalateral chambers during absorption. This suggests that certain type of active transporters is involved in the selective localization of the metabolites formed in the Caco-2 cells. The presence of MK571 substantially inhibited efflux of metabolites of EC and EGC transported, suggesting that the metabolites might be the substrates of MRP. Previous studies on the relationship between ABC transporters (P-gp and MRP) with phase I and phase II metabolic enzymes demonstrated that P-gp is capable to efflux phase I metabolites and MRP 1–3 are capable of transporting phase II metabolites such as glutathione conjugates, glucuronide conjugates, sulfate conjugates (Suzuki and Sugiyama, 2000). The metabolic enzymes and efflux transporters could cooperate to limit the entry of the EC and EGC into the cells.

Comparing to non-gallate catechins, the efflux of gallate catechin is less significant and P_{app} of gallate catechins in the secretion direction was very low and comparable to the paracellular marker, which indicated that active transcellular transport of gallate catechin was minimal. Recently, Walle's research group found the uptake of the ECG, one of the gallate catechins, into the Caco-2 cell increased in the presence of MK571 (Vaidyanathan and Walle, 2003). Therefore, MRP may also be involved during secretion transport of gallate catechin. The reason why non-gallate catechins are preferentially effluxed could be related to higher affinity of EC and EGC to the efflux transporter, which could be verified by further studies using the specific MRP transfected cell system.

In summary, the four GTC showed limited transepithelial absorption with low P_{app} values, while significant efflux mediated by MRP was found especially for non-gallated catechins during secretion. Methylation and sulfation biotransformation of GTC were the major metabolic pathways during their secretion transport across Caco-2 cell.

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