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Effect of the co-occurring components from green tea on the intestinal absorption and disposition of green tea polyphenols in Caco-2 monolayer model

Li Zhang, Moses S. S. Chow and Zhong Zuo

Abstract

This study aimed to investigate the effect of co-occurring components from green tea on the intestinal absorption and disposition of green tea polyphenols (GTPs) using the Caco-2 cell monolayer model. The absorption and secretion transport of the four GTPs, in the form of individual pure compounds, pure compound mixtures and green tea extract, were studied in the Caco-2 cell model. Four GTPs and their metabolites were analysed by HPLC/MS and HPLC coupled with electrochemical detector. The apparent permeability coefficients (P_{app}) of each compound, as well as the metabolites (mainly sulfation and methylation conjugates) generated, were compared for the different dosing formulations utilized. The results showed that the absorption transport of the four GTPs in different dosing formulations was similar. However, the secretion transport profiles of (–)-epicatechin (EC), (–)-epigallocatechin (EGC) and (–)-epigallocatechin gallate (EGCG) were altered when the GTP mixture was administered. It was suggested that transporter competition resulting in reduced efflux of EC, as well as metabolic competition resulting in reduced formation of EGC sulfate and methylated EGC sulfate, might be involved during the secretion transport of GTP mixture.

Introduction

Green tea polyphenols (GTPs), including (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG) and (−)-epigallocatechin gallate (EGCG), are the four major active components in green tea (Takehiko & Mujo 1997). Despite the well-known beneficial effects of GTPs, such as anti-carcinogenic, antioxidative and anti-platelet aggregation activity (Katiyar & Mukhtar 1996; Duffy et al 2001; Young et al 2002), the oral bioavailability of GTPs has been demonstrated to be very low both in rats (Chen et al 1997; Zhu et al 2000) and man (Warden et al 2001). In our previous study using the Caco-2 cell monolayer model, the four GTPs showed limited transepithelial absorption with low apparent permeability values and significant efflux mediated by multi-drug resistance associated protein (MRP). Methylation and sulfation biotransformation of GTPs were the major metabolic pathways during their secretion transport across Caco-2 cells (Zhang et al 2004).

Traditionally, tea infusion is prepared from tea leaves with hot water extraction, which consists of not only the four GTPs but also other co-occurring components, such as the plant matrix. Several previous studies compared the pharmacokinetic profiles of pure active components versus the extract of herbal product containing an equal amount of pure active components and demonstrated that the plant matrix in the extract might potentially alter the pharmacokinetics of the active components. Constituents in the matrix of the extract of Radix puerariae improved the uptake of daidzin in golden hamsters (Keung et al 1996). Different biliary secretion profiles of glycyrrhizin were observed when comparing oral administration of pure glycyrrhizin and aqueous liquorice extract containing glycyrrhizin (Cantelli-Forti et al 1997). The reduced oral bioavailability of glycyrrhizin in glycyrrhiza extract was discovered when comparing it with that of pure glycyrrhizin at an equivalent dose (Wang et al 1995). The extent of metabolism of isoflavonoids formononetin and biochanin A from an extract of red clover in the intestine and liver was different from that of the pure forms (Jia et al 2004). The pharmacokinetics of active compounds of hawthorn were different when administered

School of Pharmacy, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong

Li Zhang, Moses S. S. Chow, Zhong Zuo

Correspondence: Z. Zuo, School of Pharmacy, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, N.T. Hong Kong. E-mail: joanzuo@cuhk.edu.hk

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in a pure form as compared with administration in an extract form (Chang et al 2005). Therefore, the presence of other GTPs and plant matrix may also alter the intestinal absorption and disposition property of individual GTPs during their absorption across intestinal epithelium. Chow et al (2001) found that there were no significant differences in the pharmacokinetic characteristics of EGCG after oral administration of individual EGCG versus GTP extract in human subjects. However, changed pharmacokinetic behaviour of EGCG was observed when oral administration of pure EGCG and GTP extract was compared in rats, suggesting that other components in the extract might play a role (Chen et al 1997). Among the various aspects that may affect the pharmacokinetics of orally administered drugs, other co-administered components that give rise to different extent of absorption and disposition of the drugs in the small intestine should be one of the important determinants responsible for the changed pharmacokinetics of orally administered drugs. Therefore, this study was proposed to investigate the effect of the co-occurring components in green tea, such as plant matrix of tea leaves, as well as the other GTPs, on the intestinal absorption and disposition of the four major GTPs, including EC, ECG, EGC and EGCG.

Materials and Methods

Materials

EC, EGC, ECG, EGCG, lucifer yellow and MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) were purchased from Sigma Chemical Co. (USA). Isoquercitrin (IQ), serving as internal 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 GibcoBRL, Life & Technologies (USA). Phosphate-buffered saline tablets were purchased from Sigma Chemical Co. (USA).

Preparation of different dosing formulation of GTPs

Preparation of cocktail 1 solution

Cocktail 1 solution was prepared by mixing all of the pure GTPs in the PBS^+ transport buffer (pH 6.0) to reach 50 μM for each compound.

Preparation of green tea extract solution

Tea leaves (100 g) were extracted twice with 2000 mL of boiling distilled water for 30 min. The collected water extract was filtered by gauze and concentrated by Rotavaper (Büchi) to 1000 mL. The concentrated water extract was then extracted three times with an equal volume of ethyl acetate. The combined ethyl acetate layer was concentrated by Rotavaper, followed by mixing with 50 g silica gel. The mixture was then loaded onto a column packed with 80 g silica gel beforehand, then eluted with chloroform–methanol (20:1) as mobile phase to remove caffeine and other impurities. An elution with 100% methanol was conducted subsequently. The collected methanol fraction was dried by Rotavaper and the residue was re-dissolved in water. The green tea extract was obtained by freeze-drying the water solution and was stored at 4°C.

Green tea extract loading solution for the Caco-2 cell model study was prepared by dissolving green tea extract powder in the PBS^+ transport buffer (pH 6.0) to reach 276 $\mu g\,mL^{-1}$. This solution was analysed by HPLC containing 22.1 $\mu g\,mL^{-1}$ (50 μM) of ECG, 14.6 $\mu g\,mL^{-1}$ (50 μM) of EC, 35.1 $\mu g\,mL^{-1}$ (115 μM) of EGC and 125 $\mu g\,mL^{-1}$ (274 μM) of EGCG.

Preparation of cocktail 2 solution

Cocktail 2 solution was prepared by dissolving the four pure GTPs in the PBS^+ transport buffer, pH 6.0, to make the final concentration of each GTP reach the same molar concentration as that in the GTP extract solution.

Bi-directional transport studies of GTPs in the Caco-2 monolayer model

The Caco-2 monolayer model was established and the bi-directional transport studies were performed as described previously (Zhang et al 2004). Briefly, Caco-2 cells from the American type culture collection were plated onto six-well Transwell inserts at a density of 3×10^5 cell/well and cultured for 21 days before transport studies. The transport studies of cocktail 1, cocktail 2 and green tea extract were performed at the same time, using the same batch of Caco-2 cells, as that of the individual pure GTPs, which was reported previously. Samples taken from the transport study were then acidified with ascorbic acid solution (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 & Karlsson 1991).

MTT test

Caco-2 cells used for the MTT test (tetrazolium assay) were seeded into a 96-well plate at a seeding density of 5×10^4 cells/well in DMEM culture medium (Meaney & O'Driscoll 2000). The cells were cultured at 37°C for 24 h. Subsequently, the culture medium was replaced with 150 μL of green tea extract dissolved in PBS^+ (pH 6.0) at concentrations ranging from 0 to 3 mg mL^{-1}. Then the 96-well plate was incubated at 37°C for 3 h. Thereafter, 20 μL of 5 mg mL^{-1} MTT solution in PBS was added to each well and the plate was incubated for another 4 h. The solution in the wells was then removed. The remaining formazan crystals in the cells were dissolved in 200 μL of DMSO, followed by measuring its absorbance at 590 nm in a Kinetic microplate reader (Molecular Devices). The cytotoxicity

of the extract was calculated as the percentage of the absorbance relative to control.

Sample analysis

Samples taken from the transport studies of individual pure GTPs were analysed by HPLC coupled with an electrochemical detector as described previously (Zhang et al 2004). Briefly, the analytes in the samples were separated with ODS reversed-phase column (4.6 mm i.d. × 250 mm, 4.5 μm, Beckman) eluted by a mobile phase consisting of methanol, acetonitrile and phosphate buffer (pH 2.5). The samples taken from the transport studies of cocktail 1, cocktail 2 and tea extract were analysed using HPLC/MS with a detection limit of 5 ng mL^{-1} for each compound. 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) 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. ODS reversed-phase column (4.6 mm i.d. × 250 mm, 4.5 μm, Beckman) was used for separation. The HPLC 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 flow rate was set at 1 mL min^{-1}. Eluent (20%) was introduced into the mass spectrometer and the other 80% was split off.

Comparison of transport profiles of GTPs in different dosing forms

Two comparisons were performed in our study. Firstly, to investigate the potential additive effect between the four GTPs on their transport processes, comparison of the transport profile of cocktail 1 with that from individual pure GTPs (50 μM) previously reported by us (Zhang et al 2004) was performed. Secondly, comparison of the transport profile between cocktail 2 and green tea extract was conducted to study the possible effect of the other co-occurring components in the extract on the transport of the four studied GTPs.

Metabolic competition between EGC and the other GTPs

In the secretion transport studies of EGC with dosing formulations of individual pure compound, cocktail 1 and cocktail 2, samples were taken from the apical side, the receiver chamber, at the end of the experiment and were analysed by HPLC/MS. The peak area ratios of metabolites versus parent compound in the HPLC/MS chromatogram were compared among different dosing formulations to examine whether competitive metabolism was involved.

To evaluate the contribution of each GTP in the metabolic competition, the secretion transport with the following combinations was performed: 50 μM of EGC and 50 μM of EC; 50 μM of EGC and 50 μM of EGCG; 50 μM of EGC and 50 μM of ECG; 50 μM of EGC served as the control. The samples taken at the end of experiment from the receiver chambers were analysed by HPLC/MS. The peak area ratios of metabolites versus parent compound were compared between each of the above combinations and the control.

Data analysis

Reported values represent mean ± s.d. (n = 3). Statistically significant difference between two and more groups was evaluated by Student's *t*-test and analysis of variance followed by post-hoc Dunnett's test, respectively, with a significance level of $P < 0.05$.

Results

Absorption transport profile of GTPs in different dosing formulations

There was no significant difference in the P_{app} values of the absorption transport ($P_{app\ A \rightarrow B}$) of each GTP between cocktail 1 dosing form and their individual pure compound forms that had been conducted at the same time (Table 1), and reported previously by us (Zhang et al 2004), which indicated that there might not be an additive effect among the four GTPs on the absorption of each GTP. The $P_{app\ A \rightarrow B}$ of each GTP was similar for cocktail 2 and the green tea extract form (Table 1), demon-

Table 1 Bi-directional transport of the four green tea polyphenols (GTPs) in different dosing formulations

Dosing formulation	P_{app} ($\times 10^{-7}$ cm s^{-1})							
	Absorption transport (AP → BL)				Secretion transport (BL → AP)			
	EC	EGC	ECG	EGCG	EC	EGC	ECG	EGCG
Individual pure GTP (Zhang et al 2004)	1.38 ± 0.08	1.49 ± 0.13	0.96 ± 0.15	0.83 ± 0.24	29.96 ± 1.24	7.72 ± 0.44	3.86 ± 0.73	1.52 ± 0.15
Cocktail 1	1.80 ± 0.42	1.91 ± 0.33	1.17 ± 0.28	1.34 ± 0.21	25.57 ± 2.24*	11.50 ± 0.83*	3.89 ± 0.30	1.96 ± 0.14*
Cocktail 2	2.19 ± 0.23	2.14 ± 0.12	1.26 ± 0.17	1.48 ± 0.13	26.87 ± 1.32	12.61 ± 0.54	5.22 ± 0.36	3.53 ± 0.66
Green tea extract	2.10 ± 0.61	2.13 ± 0.49	1.04 ± 0.19	1.25 ± 0.26	N/A	N/A	N/A	N/A

AP, apical; BL, basolateral; N/A, not available. Data are means ± s.d., n = 3. There was no significant difference in absorption transport (Papp A → B) of each GTP between cocktail 1 and their individual polyphenols and no significant difference in absorption transport (P_{app} A → B) of each GTP between cocktail 2 and extract. *$P < 0.05$, the P_{app} values of GTPs in cocktail 1 compared with those of individual polyphenols.

strating that the matrix components also had no effect on the absorption of each studied GTP.

Secretion transport profile of GTPs in different dosing formulations

For secretion transport, the situation was much more complicated. As shown in Table 1, the P_{app} value of the secretion transport ($P_{app\ B\rightarrow A}$) of EC was slightly lower in cocktail 1 dosing form ($25.57 \pm 2.24 \times 10^{-7}$ cm s^{-1}) than that shown for the pure EC form ($29.96 \pm 1.24 \times 10^{-7}$ cm s^{-1}) (Zhang et al 2004). For EGC, its $P_{app\ B\rightarrow A}$ in cocktail 1 ($11.50 \pm 0.83 \times 10^{-7}$ cm s^{-1}) was significantly higher than that reported by us in its individual pure form ($7.72 \pm 0.44 \times 10^{-7}$ cm s^{-1}) (Zhang et al 2004). For EGCG, a marginal increase in $P_{app\ B\rightarrow A}$ for the cocktail 1 form ($1.96 \pm 0.30 \times 10^{-7}$ cm s^{-1}) was found compared with that for pure EGCG ($1.52 \pm 0.73 \times 10^{-7}$ cm s^{-1}) (Zhang et al 2004). The overall $P_{app\ B\rightarrow A}$ of ECG and EGCG were fairly low and comparable with those of the paracellular markers (P_{app} of lucifer yellow, the paracellular marker for our Caco-2 cell model = $1.63 \pm 0.12 \times 10^{-7}$ cm s^{-1}).

In the secretion transport study of green tea extract, loading the green tea extract to the basal side led to a significant drop in the transepithelial electrical resistance (TEER) value (from >600 to <150 Ωcm^2 after subtracting the background value) after only half an hour of incubation, indicating that damage to the integrity of the monolayers might have occurred. However, such damage was not observed after loading the same concentration of extract to the apical side of the chamber. In addition, MTT test with Caco-2 cells showed no cytotoxicity under the current conditions at the concentration range of 0~3 mg mL^{-1} of green tea extract. The observation might reflect that the matrix existing in the green tea extract might have displayed potential cellular toxicity in the basal side of the Caco-2 cell.

Metabolic competition between EGC and the other GTPs

Comparison of secretion transport profiles of EGC between its different dosing formulations (individual pure compound, cocktail 1 and cocktail 2) revealed that the $P_{app\ B\rightarrow A}$ values of EGC were increased remarkably in the two cocktail formulations.

The underlying mechanism was investigated further by analysing the amount of EGC metabolites formed by HPLC/MS. The major metabolites of EGC formed during its secretion transport were EGC sulfate conjugate and methylated EGC sulfate conjugate (Figure 1). Compared with EGC in individual compound form, the amount of EGC was increased in cocktail 1 and cocktail 2, whereas the formation of two metabolites of EGC declined dramatically and the methylated EGC sulfate conjugate was almost lower than the detection limit of HPLC/MS. To quantify the changes in the amount of EGC and its metabolites, the peak area ratio of metabolite to EGC was utilized as an index to represent the normalized amount of metabolite due to different loading concentrations of GTP in each formulation. Dosing with cocktail 1 reduced the peak area ratios substantially, and dosing with cocktail 2 resulted in further reduction in the peak area ratios (Figure 2). These data demonstrated that different dosing formulations could affect the formation of EGC metabolites during secretion transport.

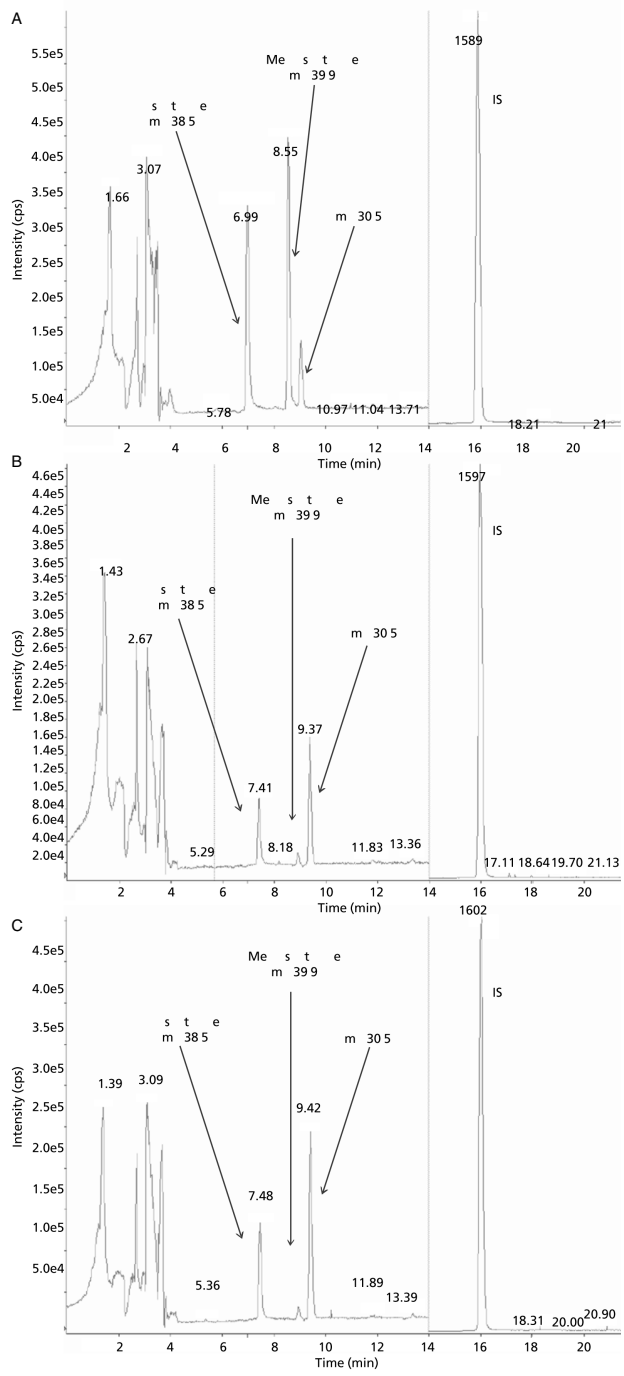

Figure 1 HPLC/MS chromatograms of EGC and its metabolites in samples taken from the apical chambers at the end of the transport experiments after basolateral loading of various EGC formulations. A. Individual pure EGC. B. Cocktail 1. C. Cocktail 2.

Our previous studies demonstrated that the four GTPs (EC, EGCG, ECG and EGC) share the same metabolic pathways

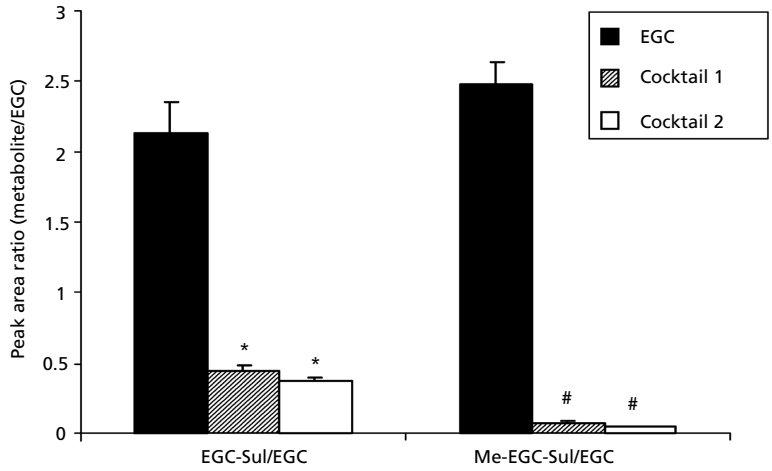

Figure 2 Effect of different dosing formulations (individual EGC, cocktail 1, and cocktail 2) on the formation of EGC metabolites during the secretion transport of EGC. Data are means + s.d., n = 3. *$P < 0.05$, compared with individual EGC; #$P < 0.05$, compared with individual EGC.

as EGC and generate mainly sulfated and methylated metabolites (Zhang et al 2004). Thus, metabolic competition between the four GTPs could result in less formation of EGC metabolites in our study.

Contribution of GTPs to the metabolism of EGC

Combination of 50 μM of EC with 50 μM of EGC reduced the formation of both EGC sulfate conjugate and methylated EGC sulfate conjugate (Figure 3 and 4). Mixture of 50 μM of ECG or EGCG with EGC mainly reduced the formation of methylated EGC sulfate conjugate, with a limited inhibition of the formation of EGC sulfate conjugate.

In summary, the above results, together with the observation from individual EGC and cocktail 1, confirmed the contribution of EC, ECG and EGCG to the competition in metabolism during secretion transport of cocktail 1.

Discussion

Previous in-vitro cell model studies discovered the efflux transport of the four-studied GTPs, which was mediated by MRP (Vaidyanathan & Walle 2001, 2003; Hong et al 2003; Zhang et al 2004). Moreover, methylation and sulfation were the major metabolic pathways for the four selected GTPs (Kuhnle et al 2000; Meng et al 2001;Vaidyanathan & Walle 2002; Lu et al 2003; Zhang et al 2004). Due to the similar efflux and metabolism mechanisms by which the four GTPs were disposed at the intestinal epithelium, potential competition may take place among the four GTPs during the oral absorption of tea infusion.

In this study, each GTP showed a similar absorption transport profile in the four studied dosing formulations, their $P_{app\ A \rightarrow B}$ values being comparable with that of the paracellular marker. The results indicated that there is neither additive effect among the four GTPs nor influence from plant matrix on the absorption transport of each of GTP.

In our previous findings, the four GTPs displayed various extent of efflux; the secretion transport rate of EC was the most rapid, followed by EGC, ECG and EGCG (Zhang et al 2004). This study showed that the secretive P_{app} value of EC in cocktail 1 decreased slightly compared with that from its individual pure form. As the four GTPs were demonstrated to be the substrates of MRP, the reduced P_{app} may be due to the competition on MRP transporter between EC and the other three GTPs in the cocktail 1 dosing form.

For secretion transport of EGC, the $P_{app\ B \rightarrow A}$ values of EGC in cocktail 1 were enhanced rather than decreased significantly compared with that from its individual pure form, which could not be explained clearly by the transporter competition. Thus, further studies were performed to monitor the relative changes in the amount of EGC and its two major metabolites (EGC sulfate and methylated EGC sulfate) using an HPLC/MS approach. As demonstrated in Figure 1, the presence of other GTPs decreased the formation of both EGC sulfate and methylated EGC sulfate. To investigate in detail the contribution of each GTP to the metabolism of EGC, the secretion transport of EGC in various combinations with other three GTPs were performed. The results confirmed that metabolic competition gave rise to more extensive efflux of EGC in the cocktail dosing forms. The mechanistic explanation is represented in Figure 5. In the secretion transport of individual pure EGC, EGC permeating into the Caco-2 cells was transformed by Phase II metabolic enzymes into EGC sulfate and methylated EGC sulfate. Both EGC and its two metabolites could be actively transported by MRP into the apical side, which was confirmed by the effect of MK 571 on the transport of EGC and its metabolites (Zhang et al 2004). In the secretion transport of cocktail 1, EGC uptake by the Caco-2 cells would compete with the other three GTPs present in cocktail 1 for the Phase II metabolic enzymes. Such metabolic competition would result in more unchanged EGC being left inside the Caco-2

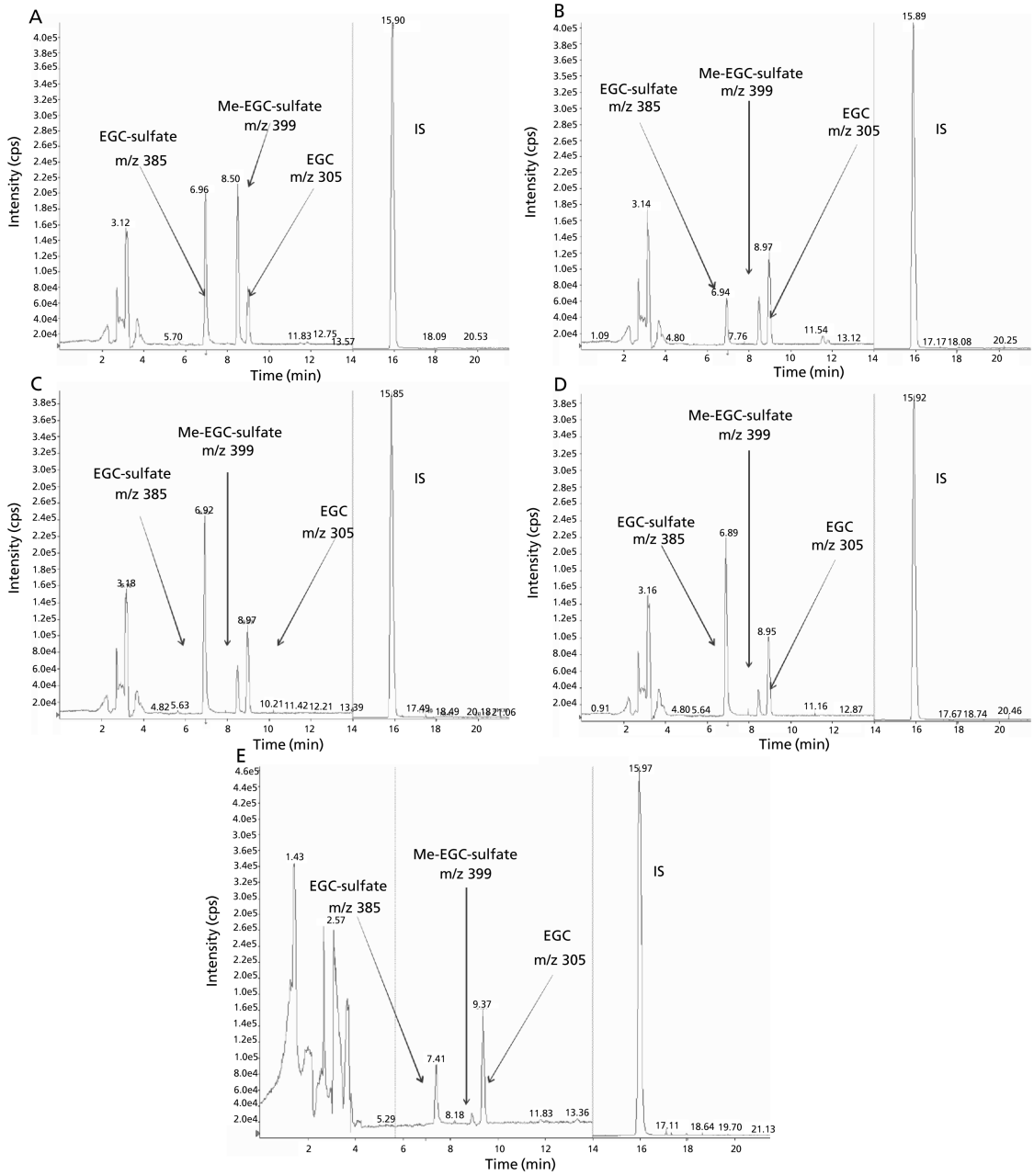

Figure 3 HPLC/MS chromatograms of EGC and its metabolites in apical samples at the end of the transport experiment after basolateral loading of various EGC combination formulations. A. Pure EGC. B. EGC + EC. C. EGC + EGCG. D. EGC + ECG. E. Cocktail 1.

cell, which would be secreted to the apical side subsequently and result in more EGC being transported to the apical side.

It seems that the integrity of the Caco-2 cell monolayer was damaged when green tea extract solution was loaded to its basal side rather than to the apical side. However, our MTT test, which involved exposure of green tea extract to the apical side of Caco-2 cells, did not show any cytotoxic effect at the studied concentration. This implied that the extract might exhibit toxicity preferentially to the basal side. It may be hypothesized that some cytotoxic substance in the extract may effectively accumulate in the Caco-2 cells via transporters located at the basal side but not at the apical side. However, further investigations are needed to support the hypothesis.

In summary, there is neither additive effect among the four GTPs nor effect from plant matrix on the absorption transport of each of GTP. However, transport as well as metabolism competition among the four GTPs may be involved in the secretion transport of the GTP.

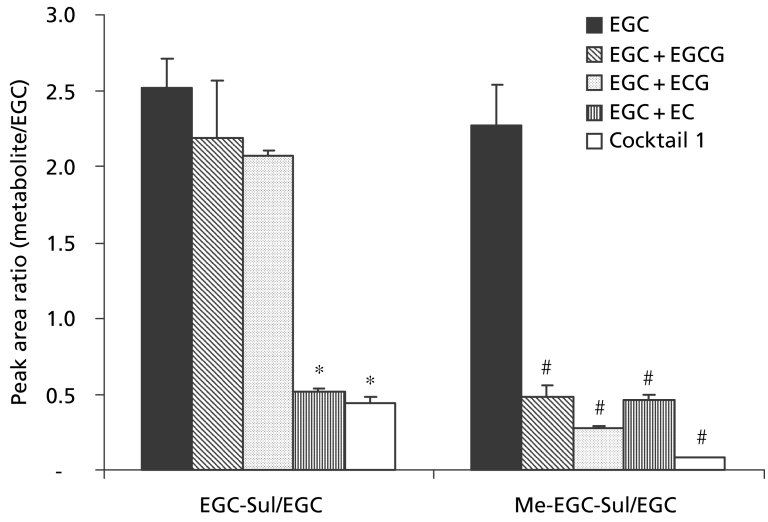

Figure 4 Effect of different combinations of EGC and other GTPs on the formation of the EGC metabolites during the secretion transport of EGC. Data are means + s.d., n = 3. *$P < 0.05$, compared with individual EGC; #$P < 0.05$, compared with individual EGC.

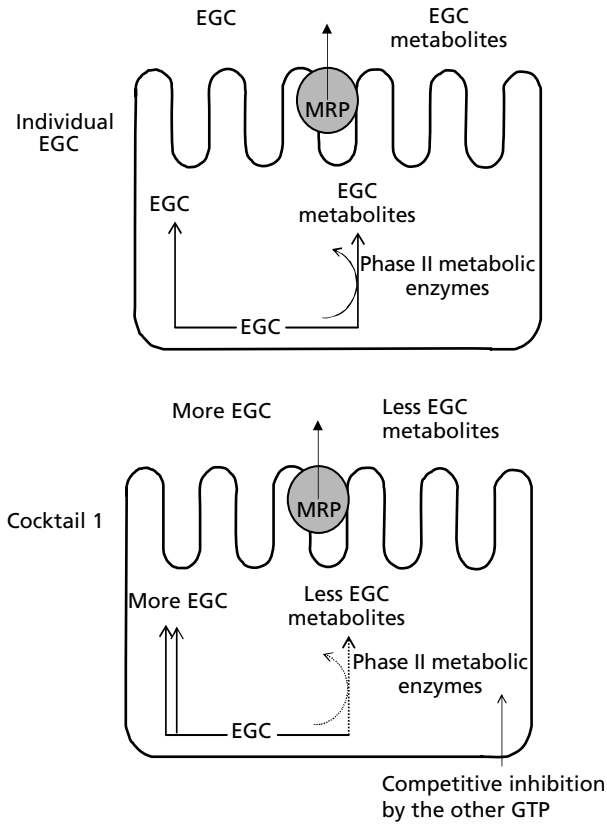

Figure 5 Schematic diagram illustrating the mechanisms of enhanced extent of efflux of EGC in various mixture formulations. Upper figure: secretion transport of EGC in individual form. Lower figure: secretion transport of EGC in cocktail 1.

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