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Metabolic inhibition and kinetics of raloxifene by pharmaceutical excipients in human liver microsomes

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ABSTRACT

This study was originally undertaken to establish the in vitro metabolic conditions and then evaluate the effect of pharmaceutical excipients (PEs) on drug metabolism in uridine diphosphoglucuronic acid-supplemented human liver microsomes. Poorly bioavailable raloxifene was chosen as a model drug. Intact drug and its two glucuronide metabolites were successfully isolated using gradient HPLC analysis and LC/MS analysis. Formation of raloxifene metabolites was affected by buffer compositions, incubation time and initial raloxifene concentrations. Under optimized metabolic conditions, 41.0% of raloxifene was converted to its metabolites after 2 h incubation. This metabolic inhibition of raloxifene by the PEs occurred in a dose-dependent manner and accordingly formed two glucuronide metabolites. In the metabolic kinetics using Lineweaver-Burk analyses, Cremophor® EL competitively inhibited formation of metabolites while sodium lauryl sulfate (SLS), polyvinylpyrrolidone K30 (PVP) and Tween® 80 significantly inhibited in a mixed competition. Although some PEs showed inhibition on glucuronidation of raloxifene in vitro, their effects on in vivo bioavailability of raloxifene need to be confirmed directly due to the dilution factors and other complicated situations influencing the bioavailability.

1. Introduction

Recent studies demonstrated that pharmaceutical excipients (PEs) may affect many physicochemical and biopharmaceutical properties of a drug when added to a dosage formulation (Azmin et al., 1986; Villalobos-Hernandez and Villafuerte-Robles, 2001; Cornaire et al., 2004; Ren et al., 2008a). It was also known that drug metabolism, mainly in gastrointestinal membranes and hepatocytes, was modulated by these PEs. For example, in one study it was shown that the hepatic metabolism of midazolam was inhibited by the surface-active excipients such as Cremophor® EL, Tween® 80 and Solutol HS 15 because the metabolizing cytochrome P450 (CYP) isoenzyme, CYP3A was prevented by these excipients (Bravo Gonzalez et al., 2004). It was also reported that poloxamer 188 and Gelucire 44/14 had potentially inhibitory effects on rabeprazole metabolism in human liver microsomes (Ren et al., 2008b). In other studies, Pluronic P85 and polyethylene glycol (PEG) 400 were found to inhibit CYP3A-mediated metabolism of verapamil in rat intestinal tissue (Johnson et al., 2002). The magnitude of this inhibitory effect depends on the characteristics of the individual drug, the quantity as well as the physicochemical properties of these PEs. For this reason, utilization of Generally Recognized As Safe (GRAS)-listed PEs has been potential strategy to modulate poorly bioavailable drugs via metabolic inhibition.

Poorly bioavailable raloxifene HCl was chosen as a model drug. Raloxifene is a selective estrogen receptor modulator that is used for the prevention and treatment of osteoporosis (Close et al., 2006). Pharmacokinetics of raloxifene are characterized by its enterohepatic circulation, high oral clearance (44 L/(kg h^{-1})) and a long plasma elimination half-life (27.7 h) (Hochner-Celnikier, 1999). However, oral bioavailability of raloxifene in humans is less than 2%, probably due to the poor absorption and most importantly, the extensive first-pass metabolism (Kemp et al., 2002; Jeong et al., 2004).

CYP and UDP-glucuronosyltransferase (UGT) are well-known enzymes that catalyze the metabolic transformation of a wide variety of drugs, thereby contributing to pre-systemic or first-pass drug metabolism (Watkins, 1992; Tukey and Strassburg, 2000). It has known that CYP enzymes play a minimal role in mediating the biotransformation of raloxifene (Morello et al., 2003). In contrast, raloxifene is known to undergo exclusive and extensive pre-systemic glucuronidation, catalyzed by UGT to form metabolites, raloxifene-4′-glucuronide or raloxifene-6-glucuronide (Fig. 1) (Lindstrom et al., 1984; Kemp et al., 2002; Jeong et al., 2004). Therefore, it seems likely that glucuronide conjugation mediated by UGT is mainly responsible for the limited bioavailability of raloxifene in

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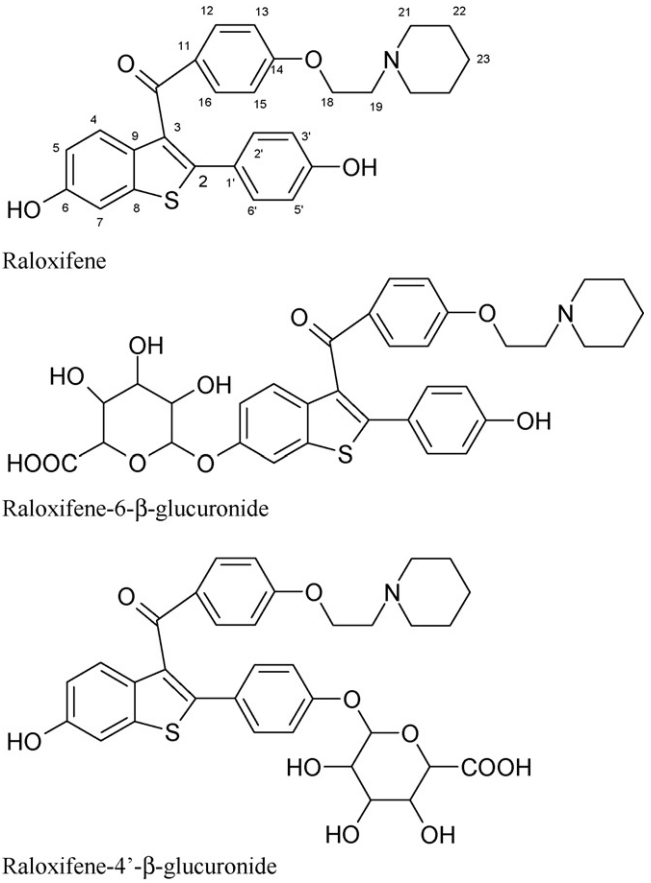

Fig. 1. Chemical structures of raloxifene and its two metabolites (raloxifene-4′-β-glucuronide and raloxifene-6-β-glucuronide).

humans. However, no studies have been done whether the PEs can modulate metabolic behavior of raloxifene kinetically.

The aim of this work was to elucidate metabolic behaviors of drug and its two metabolites in the presence of PEs using human liver microsomes after establishing in vitro metabolic conditions. The metabolic kinetics as a function of excipient concentration were also investigated. Brij® 98, Cremophor® EL, Tween® 80, poloxamer 407, sodium lauryl sulfate (SLS), PEG 6000, Gelucire 44/14 and PVP were chosen as a model excipient.

2. Materials and methods

2.1. Materials

Raloxifene was purchased from Spic Pharmaceutical Division (India). Uridine diphosphoglucuronic acid (UDPGA) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Tris (hydroxymethyl)-aminomethane was purchased from Biorad (Hercules, CA, USA). Human mixed pool microsomes were bought from In Vitro Technologies, Inc. (Baltimore, MD, USA). HPLC-grade acetonitrile was obtained from Fisher (Fisher Korea). Polyoxyethylene oleyl ether (Brij® 98), polysorbate 80 (Tween® 80), SLS and Poloxamer 407 were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO, USA). PVP and polyoxyl 35 castor oil (Cremophor® EL) were obtained from BASF (Germany). Sodium acetate trihydrate, potassium dihydrogenphosphate, PEG 6000 were purchased from Showa (Denko KK, Japan). Gelucire 44/14 was purchased from Gatteeffosse (St. Priest, France). All other chemicals were of reagent grade and were used without further purification.

2.2. HPLC analysis of raloxifene

A reverse phase HPLC system was used for the analysis of raloxifene. The HPLC system (Waters, USA) consisted of a pump (Waters™ 600 Controller), a UV–vis spectrophotometric detector (Waters™ 486 Tunable Absorbance Detector), an autosampler (Waters™ 717 plus Autosample), a degasser (Waters™ In-line Degasser), a reverse phase column (Luna 5μ C_{18} analytical column (150 mm × 4.6 mm)) and an integrator (Borwin 1.20 software).

For isocratic analysis, a mixture of 0.01 M sodium acetate (pH 4.0) and acetonitrile (at a ratio of 70:30 (v/v)) was used as the mobile phase. For gradient analysis, the ratios of the mobile phases (0.01 M sodium acetate pH 4.0 and acetonitrile) were varied to 77:23, 60:40 and 77:23 at 0, 4 and 10 min, respectively. The flow rate of mobile phase was 1 ml/min and the column elute was monitored by a UV detector set at 287 nm. The concentration of raloxifene was determined from a standard curve.

2.3. Identification of raloxifene metabolites by LC/MS

Raloxifene was separated from its possible metabolites MT1 and MT2 by HPLC method at the gradient conditions described above. Chromatographic separations were performed using a Capcell Pak C_{18} (2.0 mm × 100 mm, 3 μm) (Shisheido, Japan) with the previously described gradient except the 0.25 ml/min of flow rate. Following separation, Finnigan TSQ Quantum Ultra mass spectrometry (Thermo, USA) was performed and tuned to unit mass resolution. This was directly coupled to the HPLC system (Finnigan Surveyor, Thermo, USA) through an atmospheric pressure ionization source operated in the electrospray ionization mode. The mass spectrometer was operated in the positive ion mode, typically scanning from 200 to 1000 amu every 2 s. The capillary was operated at 250 °C, the spray voltage was set to 4.8 kV, and nitrogen was employed as a drying gas at a sheath pressure of 49 psi and an auxiliary gas pressure 25 psi.

2.4. Stability of raloxifene in buffer systems

Raloxifene was incubated with 100 mM phosphate buffer (pH 7.4) or 100 mM Tris buffer (pH 7.4) for 120 min. At post-incubation, the concentration of intact raloxifene in each buffer system was determined by isocratic HPLC analysis to assess any possible degradation of raloxifene.

2.5. Evaluation of in vitro metabolic factors

We studied the effect of several metabolic factors on the in vitro hepatic metabolism of raloxifene by enzymatic glucuronidation in liver microsomes. These factors included the types of buffer, the incubation time and the concentration of raloxifene. 25 μl microsomal solution (20 mg/mL) was diluted with 75 μl of the appropriate buffer solution and then incubated for 30 min at 37 °C to activate the microsomal enzymes. At the end of incubation, 640 μl buffer was added to the test tube followed by addition of 10 μl raloxifene (stock in methanol). The resulting reaction mixture and UDPGA (1.9 mg/mL) were separately warmed in a 37 °C water bath while shaking for 5 min. To initiate the reaction, 250 μl of warmed UDPGA was added to the reaction mixture in the warmed water bath shaking at 100 rpm. Since raloxifene is known to be extensively metabolized by glucuronidation, UDPGA was added to the reaction mixture containing liver microsomes to initiate and accelerate the glucuronidation process.

The final incubation volumes were set at 1000 μl in which the concentration of microsomes and UDPGA were 0.5 mg/mL and

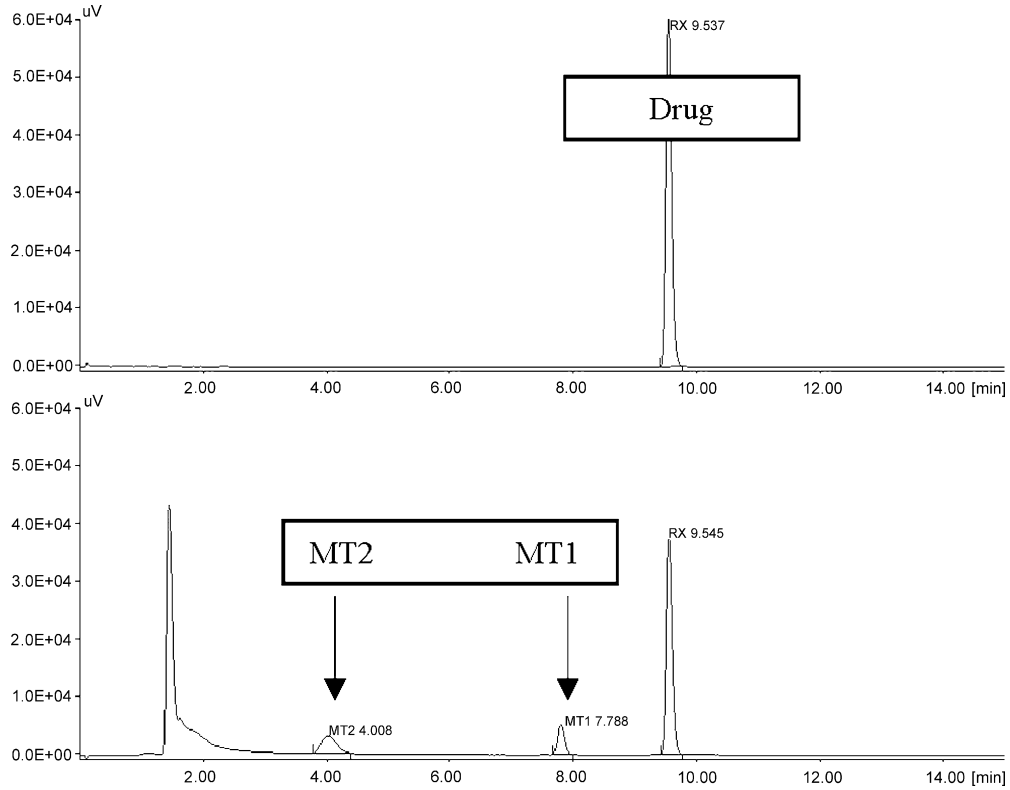

Fig. 2. Gradient HPLC chromatograms of raloxifene and its two metabolites (MT1 and MT2). Raloxifene was incubated for 60 min with microsomes (0.5 mg/mL) and UDPGA (0.475 mg/mL) in 100 mM phosphate buffer.

0.752 mM, respectively. The enzymatic reactions were terminated by the addition of 500 μl of cold acetonitrile followed by vigorously mixing the reaction sample. Finally, proteins were precipitated by centrifugation (1000 × *g*, 10 min, 4 °C) and the supernatant containing raloxifene and its metabolites were then analyzed by HPLC with isocratic condition.

2.6. Effects of PEs on metabolic behaviors of raloxifene

To study the effect of PEs on the metabolism of raloxifene in human liver microsomes, the excipients with two different concentrations (10 or 20 μg/ml) were added to the reaction mixtures together with raloxifene, and the metabolic rate of raloxifene was determined as described previously.

2.7. Metabolic kinetics

To characterize the kinetics behaviors of metabolic inhibition (competitive, noncompetitive, or uncompetitive) and to estimated K_i (inhibition constant), raloxifene (1.4–28 μM) was incubated in the presence of increasing PE concentration (5–40 μg/ml). To further characterize this potential metabolic interaction, Lineweaver-Burk analyses were carried out in order to determine the nature of the inhibition. K_i values, the values of inhibitor constants, were determined by regression analysis of secondary plots (K_m/V_m ratio as a function of inhibitor concentration).

$$\frac{K_{m\,app}}{V_{max\,app}} = \frac{K_m[I]}{K_i.V_{max}} + \frac{K_m}{V_{max}}$$

where the values of K_m and V_{max} are Michaelis–Menten parameters (K_m and V_{max}) by non-linear regression analysis, and the values of $K_{m\,app}$ and $V_{max\,app}$ represent the values of K_m and V_{max} in the presence of PEs [*I*]. The K_i value was determined from the intercept on the *X*-axis line (Bourrié et al., 1996; Kakkar et al., 1999; Ubeaud et al., 1999).

2.8. Statistical analysis

All data were presented as mean ± standard deviation (SD). The statistical significance of differences was performed using analysis of variance (ANOVA) test and then assessed by a Duncan's multiple range test. A probability level at 1% or 5% was considered to be statistically significant.

3. Results and discussion

3.1. Identification of raloxifene and its metabolites

The drug peak was clearly observed without any interference from the microsomes and UDPGA solution. When raloxifene metabolism was induced by incubating raloxifene with human liver microsomes supplemented with UDPGA, two new peaks corresponding to MT1 and MT2 appeared at 2.1 and 3.3 min, respectively (data not shown) due to the formation of drug metabolites. Furthermore, the peak areas of two metabolites (MT1 and MT2) increased but the raloxifene peak areas concurrently decreased as the incubation time increased. These data suggest that two metabolites peaks are potential metabolites of raloxifene catalyzed by UDPGA.

Although the isocratic HPLC system could resolve the drug and metabolites, the retention times of two metabolites was too close. The gradient HPLC chromatograms of raloxifene and its two metabolites (MT1 and MT2) are shown in Fig. 2. The drug and two metabolites were well resolved and the retention times of MT2, MT1 and raloxifene were 4.0, 7.8 and 9.5 min, respectively.

To gain a closer insight into the chemical identity of the two metabolites (MT1 and MT2), the eluted metabolites solutions col-

lected from the gradient HPLC analysis were subjected to the electrospray ionization mode mass spectrometry with scan ion monitoring. The mass spectra of raloxifene and its two metabolites (MT1 and MT2) are given in Fig. 3. Mass spectra demonstrated that the molecular weights of MT1 and MT2 (MH^+ $m/z = 650$) were 176 units higher than that of raloxifene (MH^+ $m/z = 474$, where m/z presents as mass to charge ratio). UGT enzymes can be categorized into two families: UGT1 and UGT2. The UGT2 family is engaged in only a few pharmacologically active glucoronide conjugates. UGT1 traditionally has been recognized as the isoform

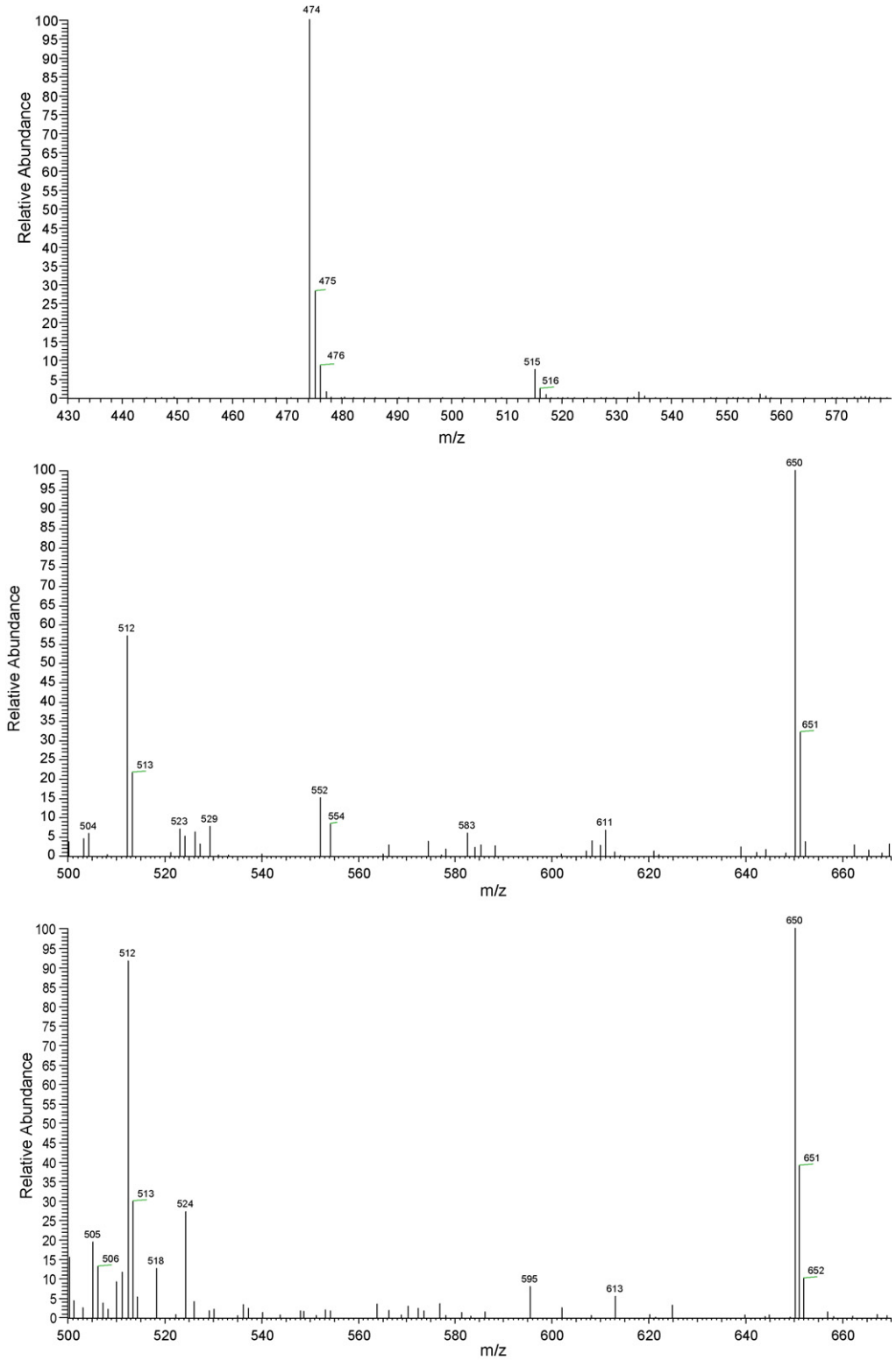

Fig. 3. The mass spectra of raloxifene and its two metabolites (MT1 and MT2). The two metabolites solutions were collected from HPLC and then analyzed by the MS with an electrospray ionization mode. Raloxifene(top), MT1(middle) and MT2(bottom).

responsible for the glucuronidation of various compounds like bilirubin, estradiol (UGT1A1), phenols, acetaminophen (UGT1A6) and carcinogenic amines (UGT1A9) (Zamek-Gliszczynski et al., 2006). The formation of raloxifene glucuronide isomer from human liver microsome incubation in this study are in agreement with a previous report showing hepatic UGT1A1 as a primary catalyst of raloxifene metabolic clearance via the 6′-glucuronide pathway (Kemp et al., 2002). Therefore, our data strongly suggests the possibility that MT1, MT2 are a 4′ or 6-glucuronide conjugate of raloxifene, formed by glucuronidation mediated by UGT present in the liver microsomes.

3.2. Establishment of in vitro metabolic conditions

To determine whether raloxifene is chemically degraded in microsome-free buffer systems, raloxifene (5 and 10 μg/mL) was incubated with phosphate or Tris buffer. Under our experimental conditions, raloxifene remained intact in both buffer systems during incubations up to 120 min (>99.0%) and there was no chemical degradation of raloxifene in microsome-free buffer systems during incubation. Therefore, both Tris and phosphate buffer (pH 7.4) could be readily used for the subsequent metabolic studies.

The activity of enzymes responsible for in vitro metabolism of drugs can be affected by numerous factors such as buffer composition, pH and the ionic strength of the assay mixture (Green and Tephly, 1996; Kurkela et al., 2003). According to previous reports, the addition of alamethicin, bovine serum albumin or Mg^{2+} may act to disrupt the membrane of the endoplasmic reticulum (Meech and Mackenzie, 1997; Trapnell et al., 1998; Kemp et al., 2002). Therefore, we investigated whether the activity of enzymes mediating the raloxifene metabolism in human liver microsomes were affected by the buffer systems in the reaction mixtures. The effect of buffer compositions on raloxifene metabolism in human liver microsomes is shown in Fig. 4A. The intact drug concentration decreased more in Tris buffer than in phosphate buffer. The addition of $MgCl_2$ to the buffer systems resulted in a further decrease of the intact drug concentration. The % decrease in the intact drug concentration in the Tris buffer was 22.1, 39.5 and 42.7%, respectively when 0, 5 and 10 mM $MgCl_2$ was added. These data suggested that the metabolic reaction would be more desirable to improve enzymatic activity in human liver microsomes when the Tris buffer containing Mg^{2+} was used. It would seem that these conditions may allow UGT's active sites to be more available for catalyzing glucuronidation. Therefore, 100 mM Tris–HCl buffer (pH 7.4, 5 mM $MgCl_2$) was used for subsequent metabolic studies.

The incubation time is one of the crucial factors for drug metabolism. The effect of incubation time on metabolic behaviors of raloxifene in human liver microsomes is shown in Fig. 4B. The intact raloxifene concentration gradually decreased as a function of incubation time. At the same time, the concentration of the two major drug metabolites (MT1 and MT2) increased as measured by the peak areas. These data suggest that the percentage decrease in the concentration of intact raloxifene is also an index of drug metabolism catalyzed by UDPGA.

3.3. Metabolic modulation by PEs

The effect of various PEs on raloxifene metabolism in human liver microsomes is shown in Fig. 5. Addition of PEs could differentially increase the solubility of raloxifene in the reaction mixture (data not shown). However, the effect of various PEs on raloxifene metabolism was not due to the difference in raloxifene solubility in each system since the raloxifene concentration was always maintained below the saturation solubility of raloxifene. In the absence of any excipients, 59% of raloxifene remained intact after

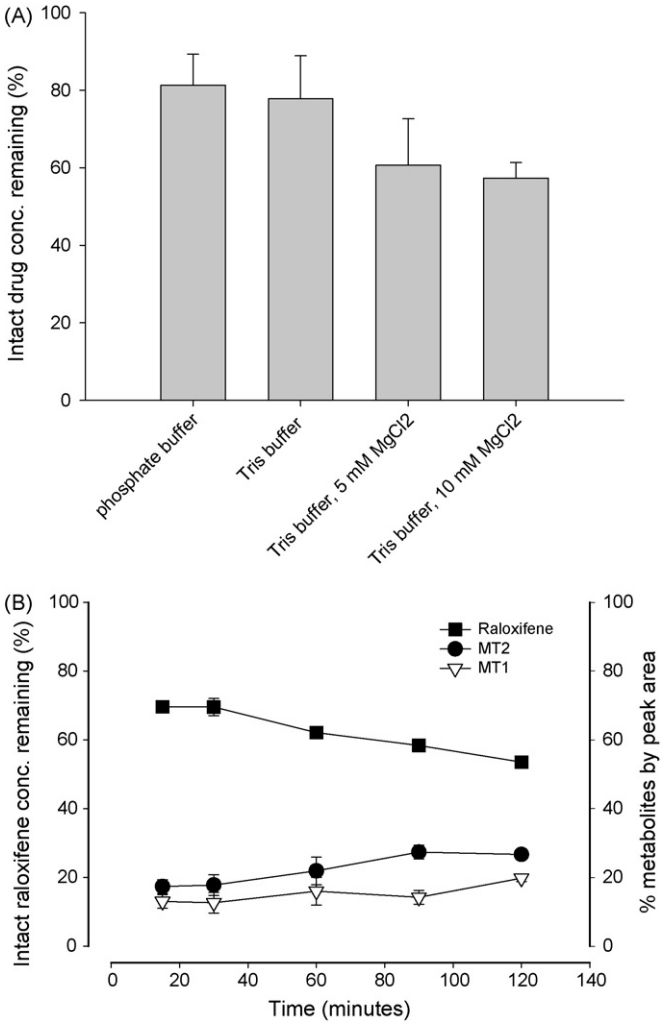

Fig. 4. Effect of buffer compositions on raloxifene (5 μg/ml) metabolism for 60 min incubation (A), and the effect of incubation time on raloxifene concentration and formation of its two metabolites in 100 mM Tris–HCl buffer (pH 7.4, 5 mM $MgCl_2$) (B) in human liver microsomes (0.5 mg/mL) containing UDPGA (0.475 mg/mL).

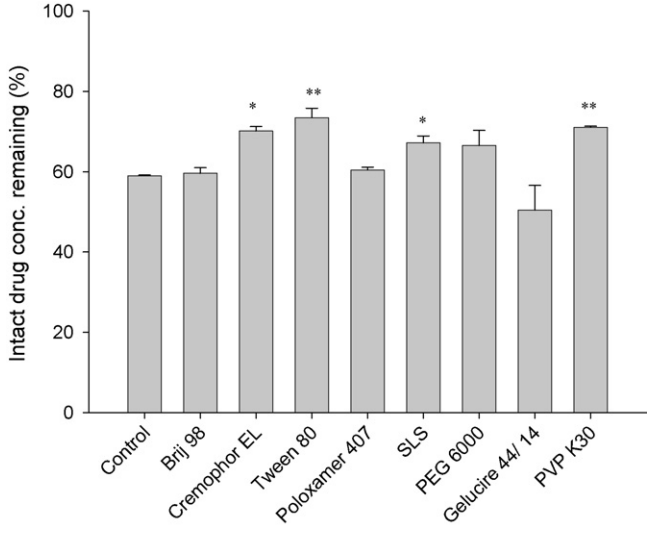

Fig. 5. Effect of PEs (20 μg/mL) on raloxifene metabolism in human liver microsomes. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control.

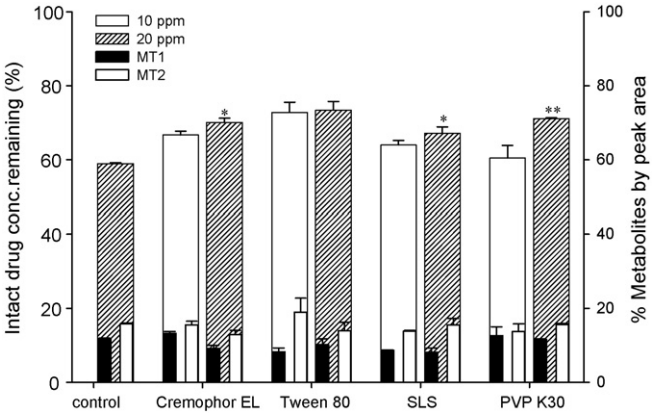

Fig. 6. Dose-dependent metabolic inhibition of drug and formation of two metabolites (MT1, MT2) by four PEs (10 or 20 μg/mL concentrations). $^{*}p < 0.05$, $^{**}p < 0.01$ compared with 10 μg/mL concentrations.

2 h incubation, suggesting that approximately 41% of the drug was converted to its metabolites. 20 μg/ml of Poloxamer 407, PEG 6000 and Gelucire 44/14 did not change metabolism of raloxifene statistically. Interestingly, Cremophor® EL, Tween® 80, PVP K30 and SLS significantly inhibited drug metabolism as compared with control ($p < 0.05$), indicating that these excipients were effective in inhibiting raloxifene metabolism in liver microsomes supplemented with UDPGA.

Similar metabolic inhibition was also observed when the concentration of the excipients was reduced to 10 μg/ml. Dose-dependent metabolic inhibition of drug and formation of two metabolites (MT1, MT2) by the PEs (10 or 20 μg/ml) was also observed (Fig. 6). The inhibition of drug metabolism was significantly increased as the concentration of PEs was double. The addition of these PEs also resulted in decreasing formation of the two metabolites as compared to control, based on peak areas of formed metabolites.

The molecular interaction of the raloxifene and raloxifene metabolites in the presence of PEs was characterized using ^{1}H NMR spectra in Fig. 7. NMR data of raloxifene and its two metabolites were consistent with the literature values for proton chemical shifts, protein integral ratios and coupling patterns (Kemp et al., 2002). The absence of shift or intensity change in ^{1}H NMR spectra of raloxifene with PEs corresponding to H4, H5, H7, H12, H13, H15, H16, H2′, H3′ H5′, H6′ proton positions indicated that PEs did not interact with raloxifene directly. Collectively, the PEs could alter the conformation of the active sites and inhibit the UGT activity via competitive or mixed-type inhibition mechanism in the presence of raloxifene.

PEs are traditionally known as inert but recent studies have evaluated the effect of PEs as solubilizers, permeation enhancers, and stabilizers as well as absorption facilitators (Thanou et al., 2001; Izutsu and Kojima, 2002; Tirucherai and Mitra, 2003; Tian et al., 2006). As a metabolic inhibitor, Tween® 80 has been shown to decrease CYP activity by interacting with the microsomal protein, altering its conformation and, consequently destroying its function (da Silva and Meirelles, 2004). It had been reported that addition of Cremophor® EL, Tween® 80, Solutol® HS prevented CYP3A mediated metabolism of midazolam by interacting with biological membranes (Bravo Gonzalez et al., 2004). High concentrations of surface active agents such as Triton X-100 and Lubrol PX, inhibit the quaternary ammonium-linked glucuronidation of chlorpromazine and imipramine catalyzed by expressed human UGT (Thomassin et al., 1985; Green and Tephly, 1996). Brij® 58 and Lubrol 12A9 released small amounts of protein and also some phospholipids

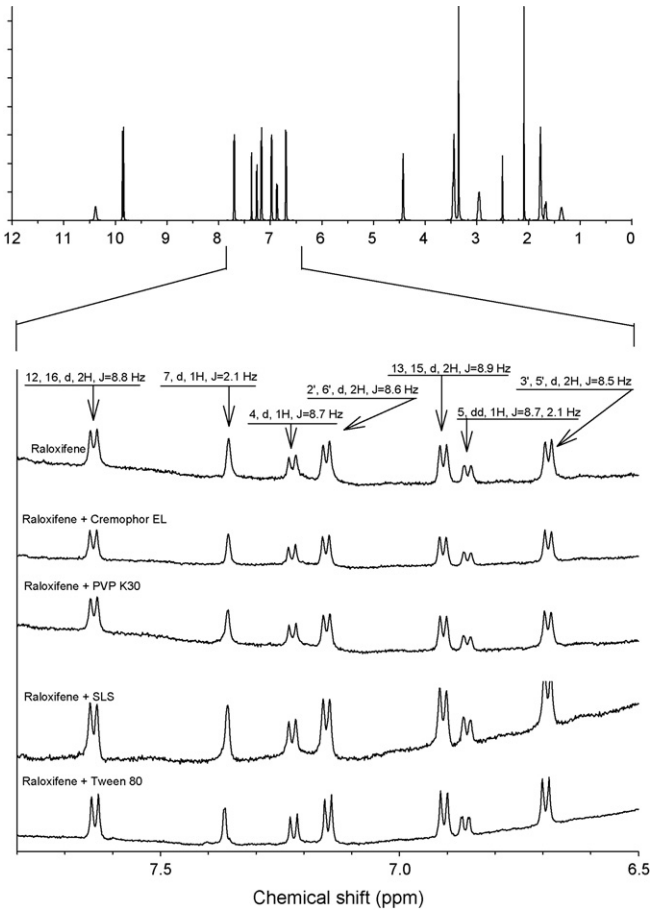

Fig. 7. ^{1}H NMR spectra of raloxifene (20 μg/mL) in the presence of PEs (40 μg/mL).

from the membrane (Illing and House, 1981). Surface active agents may hinder UGT by disrupting their interaction with the phospholipids that are necessary for catalytic activity (Kurkela et al., 2003).

Our data suggest that Cremophor® EL, SLS, PVP K30 and Tween® 80 are potential inhibitors of the hepatic metabolism of raloxifene. Inhibitory effects of the PEs on drug metabolism appeared to involve drug solubility, micellar formation and/or direct disruption of enzyme activities as described previously (Bravo Gonzalez et al., 2004; Ren et al., 2008a,b). However, these potential excipients might be more effective in blocking the interaction of UGT with phospholipids than other excipients, thereby exerting UGT inhibitory activity because drug concentration used was under the solubility limit and the PEs were below the critical micelle concentration. The dose-dependent inhibition of drug metabolism by these excipients was also motivated in dosage form design. Inclusion of these PEs in raloxifene dosage formulations may provide an effective means to decrease the first pass metabolism in gastrointestinal tract and liver, thereby improving raloxifene's oral bioavailability. However, the detailed mechanism of drug and PEs interaction at the molecular levels should be needed to test this hypothesis how these PEs may reach the sites of the metabolic enzymes, presumably both in the intestine and/or hepatic cells.

3.4. Metabolic kinetics

To understand the metabolic inhibition of four PEs: Cremophor® EL, Tween® 80, PVP K30 and SLS, metabolic kinetics as a function of excipient concentration was investigated. The reaction rates were calculated as the amount of raloxifene reacted per

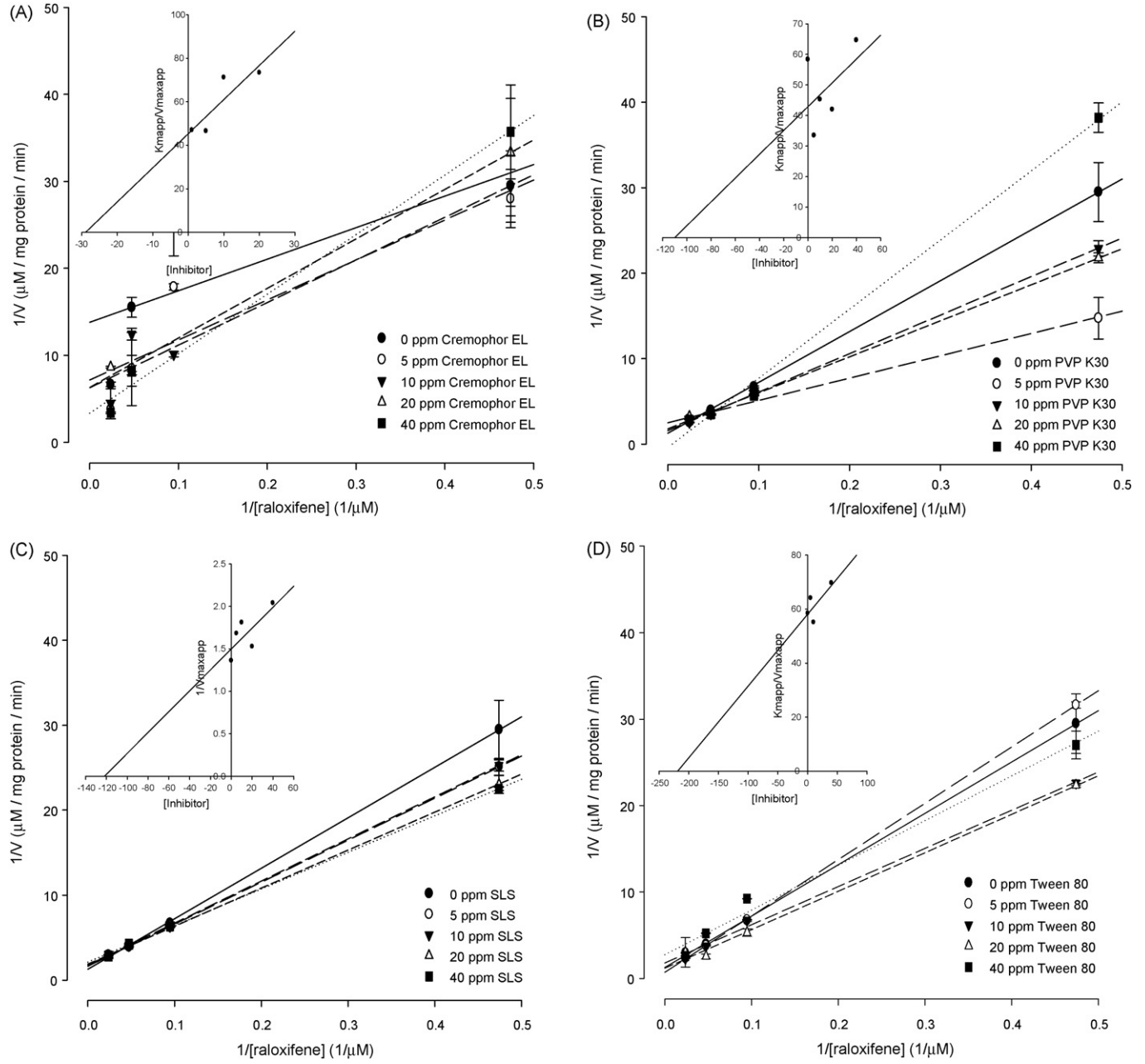

Fig. 8. Metabolic kinetics of raloxifene in the presence of PEs using Lineweaver-Burk plot and secondary conversion plot (inserted box). Cremophor® EL (A), PVP K30 (B), SLS (C), and Tween® 80 (D).

minute of incubation time and per milligram of microsomal protein. Michaelis–Menten parameters, K_m and V_{max} for raloxifene were 219 μM and 1.2 μM/mg protein/min, respectively.

Fig. 8 depicts metabolic kinetic profiles of raloxifene in the presence of PEs using Lineweaver-Burk plot. As Cremophor® EL concentration increased, metabolic inhibition of raloxifene was observed in a concentration-dependent manner (Fig. 8A). The intercept on the *X*-axis line, the K_m value, varied whereas the V_{max}, the intercept on the *Y*-axis line was not affected statistically. This is consistent with a competitive-type inhibition between Cremophor® EL and raloxifene at the level of the active site. Whereas, in the presence of increasing concentration of PVP K30, SLS and Tween® 80, an increase in the K_m value and a decrease in the V_{max} value was investigated, respectively. This is consistent with a mixed-type inhibition (Fig. 8B–D). The value of K_i, the inhibition constant, would occupy 50% of the binding sites. The K_i value of Cremophor® EL, PVP K30, SLS and Tween® 80 was computed at approximately 29, 110, 190 and 218 ppm, respectively (Fig. 8A–D, inserted box), indicating a high affinity for enzyme for raloxifene metabolism. This study shows that PEs have the potentials to inhibit in vitro metabolism of raloxifene in human liver microsomes in a different mechanism.

4. Conclusions

We have established in vitro metabolic systems in human liver microsomes. The two major metabolites formed from the metabolic reaction of raloxifene with UDPGA-supplemented liver microsomes appear to be glucuronide conjugates of raloxifene, as revealed by gradient HPLC chromatogram and LC mass spectra.

Raloxifene metabolism in liver microsomes was affected by buffer composition, incubation time and initial raloxifene concentration. It was also demonstrated that incorporating PEs readily inhibited raloxifene metabolism via competitive inhibition or mixed-type inhibition mechanism. These in vitro metabolic behaviors could be applicable in the selection of proper PEs at the early formulation stages. In addition, Utilization of appropriate PEs in dosage formulations provides a way to modulate bioavailability of poorly bioavailable drug like raloxifene. However, the effects of some PEs on in vivo bioavailability of raloxifene need to be confirmed by in vivo experiments directly.

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