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博士學位論文

EFFECTS OF QUERCETIN AND MORIN ON THE BIOAVAILABILITY OF DOXORUBICIN IN RATS

朝鮮大學校 大學院

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흰쥐에서 퀠세틴과 모린이 독소루비신의 생체이용율에 미치는 영향

2009 年 2 月 25 日

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이 論文을 藥學博士學位申請 論文으로 提出함.

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CONTENTS

Part I. Effect of Quercetin on the Bioavailability of Doxorubicin in Rats

Part II. Effect of Morin on the Bioavailability of Doxorubicin in Rats

LIST OF TABLES

LIST OF FIGURES

Abstract

Effects of quercetin and morin on the pharmacokinetics of doxorubicin in rats

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Doxorubicin (DOX), an anthracycline antibiotic, possesses broad-spectrum antineoplastic activity, and is one of the most important anticancer agents. The purpose of this thesis is to investigate the effects of flavonoids (quercetin and morin) on the bioavailability or pharmacokinetics of DOX in rats. Thus, DOX was administered intravenously (i.v.; 10 mg/kg) or orally (p.o.; 50 mg/kg) without or with oral quercetin (0.5, 3 and 10 mg/kg) or morin (0.5, 3 and 10 mg/kg).

In the presence of quercetin (0.5, 3 and 10 mg/kg) and morin (0.5, 3 and 10 mg/kg), the total area under the plasma concentration–time curve from time zero to time infinity (AUC) and the peak plasma concentration (C_{max}) of oral DOX were significantly greater and higher, respectively, than those of without flavonoids. Consequently, the extent of absolute oral bioavailability (F) of DOX in the presence of quercetin was considerably greater than that without quercetin. Compared to the i.v. control, the presence of flavonoids increased bioavailability of i.v. DOX. The enhanced bioavailability of oral DOX by oral flavonoids may be due to the inhibition of both P-glycoprotein (P-gp) and cytochrome P450 (CYP) 3A subfamily in the intestine and/or liver by flavonoids.

This result may suggest that the development of oral DOX combination with flavonoids is feasible, which is more convenient than the i.v. dosage forms. Furthermore, since the present study raised the awareness about the potential drug interactions by concomitant use of DOX with flavonoids (quercetin and morin), the dosage regimen of DOX should be

taken into consideration, if this result is confirmed in clinical studies.

Key words Bioavailability; Pharmacokinetic; DOX; flavonoids; Quercetin; Morin; P-gp; CYP3A subfamily; Rats

국 문 초 록

흰쥐에서 퀠세틴과 모린이 독소루비신의 생체이용율에 미치는 영향

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독소루비신 (doxorubicin)은 엔트라사이클린계 항암제로서 (anthracycline antibiotic) 광범위한 항종양효과를 갖고 있어 널리 사용되고 있다. 본 학위논문에서는 플라보노이드인 퀠세틴과 모린을 각각 독소루비신과 동시투여 했을때 독소루비신의 생체이용율에 미치는 영향을 연구 검토하였다. 독소루비신을 경구 (50 mg/kg) 또는 정맥 (10 mg/kg)으로 퀠세틴 (0.5, 3 및 10 mg/kg) 또는 모린 (0.5, 3 및 10 mg/kg)과 동시에 투여하였다. 퀠세틴과 모린 투여군에서 독소루비신의 혈장농도-시간곡선하면적 (AUC)과 최고혈중농도 (C_{max})는 대조군에 비해 현저히 증가되었다. 대조군에 비해 플라보노이드 투여군에서 정맥으로 투여된 독소루비신의 생체이용율은 증가되었다. 본 연구에서 경구투여 한 독소루빈의 생체이용율이 증가 된 것은 경구투여 한 플라보노이드류인 퀠세틴과 모린이 P-당단백질 (P-gp) 및 간대사효소인 사이토크롬 P450 [cytochrome P450 (CYP) 3A]를 억제한 결과라고 사료된다.

본 연구에서 플라보노이드인 퀠세틴과 모린을 각각 항암제인 독소루비신과 투여시 경구투여 된 독소루비신의 생체이용율을 유의성 있게 증가시켰다. 독소루비신을 경구투여하면 정맥투여보다 환자에게 편리하다. 본 연구결과를 토대로, 임상에서 플라보노이드인 퀠세틴과 모린이 독소루비신에 미치는

영향을 연구검토하는 것이 바람직하다고 사료된다.

Part I. Effect of Quercetin on the Bioavailability of Doxorubicin in Rats

Abstract

Doxorubicin (DOX), an anthracycline antibiotic, possesses broad-spectrum antineoplastic activity, and is one of the most important anticancer agents. The purpose of this thesis is to investigate the effects of quercetin, the antioxidant, on the bioavailability or pharmacokinetics of DOX in rats. Thus, DOX was administered intravenously (i.v.; 10 mg/kg) or orally (p.o.; 50 mg/kg) without or with oral quercetin (0.5, 3 and 10 mg/kg).

In the presence of quercetin, the total area under the plasma concentration–time curve from time zoro to time infinity (AUC) and the peak plasma concentration (C_{max}) of oral DOX were significantly greater than those of without quercetin. Consequently, the extent of oral bioavailability (F) in the presence of quercetin was considerably greater (28.6%, 86.3% and 144% increase for 0.5, 3 and 10 mg/kg of quercetin, respectively) than that without quercetin. In the presence of quercetin, the AUC of i.v. DOX was significantly ($p < 0.05$) greater (35.0% increase for 10 mg/kg of quercetin) than that without quercetin. The CL of i.v. DOX was significantly ($p < 0.05$) decreased in the presence of quercetin (20.6% and 22.5% decrease for 3 and 10 mg/kg of quercetin, respectively) than that without quercetin. The enhanced bioavailability of oral DOX by oral quercetin may be due to the inhibition of both P-glycoprotein (P-gp) and cytochrome P450 (CYP) 3A subfamily in the intestine and/or liver by quercetin.

This result may suggest that the development of oral DOX combination with quercetin is feasible, which is more convenient than the i.v. dosage forms. Furthermore, since the present study raised the awareness about the potential drug interactions by concomitant use of DOX with quercetin, the dosage regimen of DOX should be taken into consideration, if this result will be confirmed in clinical studies.

Key words Bioavailability; DOX; Quercetin; P-gp; CYP3A subfamily; Rats

1. Introduction

Many researchers have attempted to circumvent inhibition of P-glycoprotein (P-gp) during cytotoxic drug administration. For example, P-gp inhibitors, such as verapamil, cyclosporine, valspodar, GF120918 or LY357739 have previously been used to enhance intracellular accumulation of drugs in the multidrug resistance (MDR) cells (Avendano *et al*., 2002; Gottesman *et al*., 2002). P-gp, an important member of the ATP binding cassette (ABC) family which effluxes substrates out of cells, is highly expressed in solid tumours of epithelial origin, such as the colon (Cordon-Cardo *et al*., 1990) kidney (Fojo *et al*., 1987) and breast (Merkel *et al*., 1989). Cytochrome P450 (CYP) 3A subfamily, a major phase I drug metabolizing enzyme, are co-localized with P-gp in the liver and intestine (Wang *et al*., 2001; Fakhoury *et al*., 2005). Thus, a combined role of P-gp and CYP3A subfamily could decrease oral bioavailability of drugs which are substrates of P-gp and CYP3A subfamily.

Doxorubicin (DOX), an anthracycline glycosidic anticancer drug, impairs DNA synthesis during tumor cell division. It is most commonly used for the treatment of lymphoma, osteosarcoma and other sarcomas, carcinomas, and melanoma (Schwarzbach *et al*., 2002; Langer *et al*., 2006; Lind *et al*., 2007; Lundberg *et al*., 2007; Smylie *et al*., 2007). It is metabolized to doxorubicinol and the aglycones, doxorubicinone and 7-deoxydoxorubicinone (Speth *et al*., 1988) (Figure 1). Doxorubicinol is cytotoxic but the aglycones are not. The chronic phase of DOX toxicity is probably mediated by preferred metabolic conversion of DOX to doxorubicinol (Minotti *et al*., 2004). The DOX metabolism to doxorubicinol occurs by cytoplasmic NADPH-dependent aldose, aldehyde, and carbonyl reductases. The main mechanism of doxorubicinol toxicity is its interaction with iron and subsequent formation of reactive oxygen species (ROS) affecting biomacromolecules (Speth *et al*., 1988; Minotti *et al*., 2004). Doxorubicinol is also transformed into aglycones (Mross *et al*., 1988). DOX is a substrate of P-gp (Gustafson *et al*., 2005). The P-gp is co-localized with CYP3A4 in small intestine, thus, P-gp and CYP3A4 may act synergistically for the presystemic drug metabolism and lead to the prolonged exposure of P-gp substrates to CYP3A4, resulting in the limited absorption of drugs (Gottesman *et al*., 1993; Gan *et al*., 1996; Wacher *et al*., 1998; Kusuhara *et al*.,

1999; Wacher *et al*., 2001;).

Flavonoids are the most abundant polyphenolic compounds present in the human diet, such as fruits, vegetables, plant-derived beverages, such as tea, and red wine. Flavonoids have a variety of beneficial pharmacological properties, including antitumor, antioxidation, antiviral, and anti-inflammatory activities (Middleton *et al*., 2000). On the other hand, flavonoids are reported to modulate CYP3A4 or P-gp (Lee *et al*., 1994; Chieli *et al*., 1995; Di Pietro *et al*., 2002).

Quercetin (Figure 2) is a member of an extensive class of polyphenolic flavonoid compounds that is widely distributed mainly as glycosides in components of the daily diet such as onions, apples, berries, tea and red wine (Hertog *et al*., 1992; Hertog *et al*., 1995). Currently, quercetin is in clinical trial as an anticancer therapy and is a potential drug of the future (Ferry *et al*., 1996). Epidemiological studies in the U.S., Europe, and Asia have estimated that the daily dietary intake of quercetin by an individual ranges from 4 to 68 mg (Hertog *et al*., 1993; Hertog *et al*., 1995; Rimm *et al*., 1996; Knekt *et al*., 1997), and even can be as high as several 100 mg in dietary supplements and several grams in anticancer therapy (Lamson and Brignall, 2000). It has been reported that quercetin could competitively inhibit the members of MDR family, P-gp, the multidrug resistance associated proteins 1 (MRP1) and the breast cancer resistance protein (BCRP) (Scambia *et al*., 1994; van Zanden *et al*., 2005; Cooray *et al*., 2004), and the metabolizing enzyme, CYP3A4 (Guengerich and Kim, 1990; Miniscalco *et al*., 1992). Dupuy *et al*. (2003) reported that the plasma concentration–time curve from time zoro to time infinity (AUC) and plasma concentrations of moxidectin (a substrate for P-gp and CYP3A) was greater and higher, respectively, when used concomitantly with 10 mg/kg of quercetin in lambs.

Several flavonoids have been shown to be able to increase accumulation of anticancer drugs in resistant human cancer cells. Quercetin and its methoxylated derivative inhibited the efflux of rhodamine-123 and restored sensitivity to DOX in MCF-7 breast cancer cells (Scambia *et al*., 1994).

The antioxidant properties of flavonoids and their ability to chelate free iron could also be effective in reducing the cardiotoxicity of DOX. Orally administered quercetin, as a P-gp and CYP3A inhibitor, may provide anticancer effects to improve the bioavailability of DOX in combination therapy. Therefore, the purpose of this thesis is to examin the effects

of quercetin on the bioavailability and pharmacokinetics of DOX in rats.

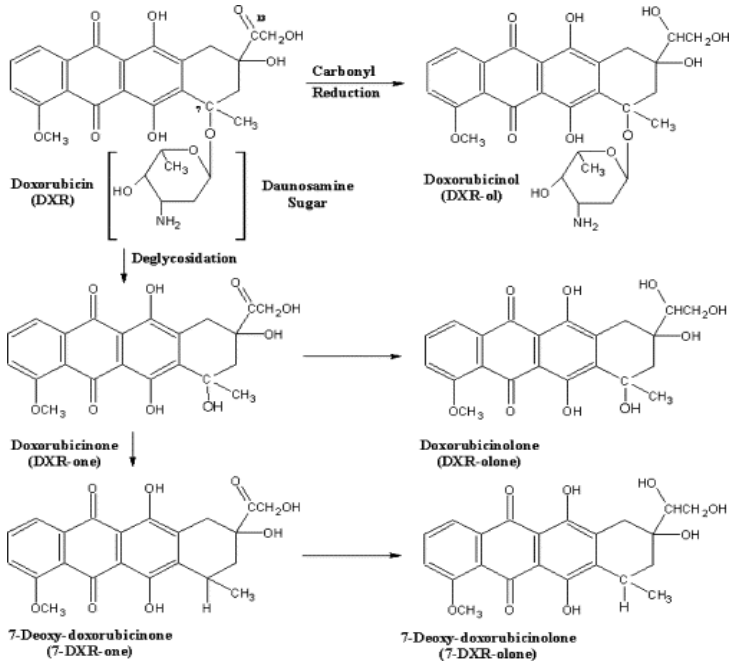

Figure 1. Chemical structures of DOX and its main metabolites.

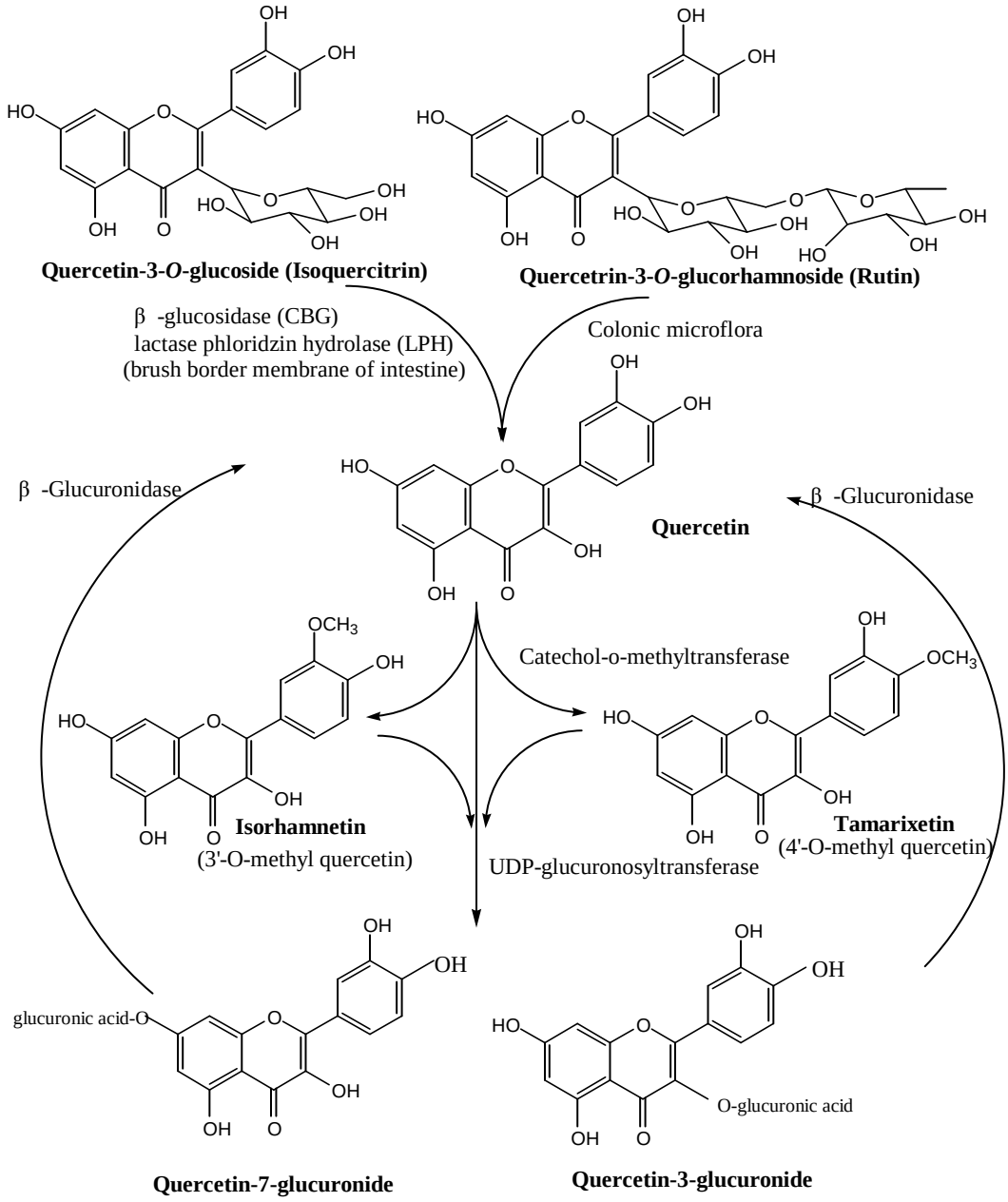

Figure 2. Biotransformation of quercetin (glucosides) via gastrointestinal system (Structures of quercetin, isoquercitrin and rutin, and the methylated quercetin metabolites, isorhamnetin and tamarixetin).

2. Materials and methods

2.1. Chemicals

DOX was obtained from Boryung Pharmaceutical Co. (Seoul, Republic of Korea). Quercetin and daunorubicin [internal standard for the high-performance liquid chromatographic (HPLC) analysis of DOX] were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Methanol and acetonitrile (HPLC grade) were products from Merck Co. (Darmstadt, Germany). Other chemicals were of reagent or HPLC grade.

2.2. Drug administration

The protocols of the animal studies were approved by Animal Care Committee of Chosun University (Gwangju, Republic of Korea). Male Sprague–Dawley rats (7–8 weeks, weighing 270–300 g) were purchased from Dae Han Laboratory Animal Research Co. (Eumsung, Republic of Korea). They were given free access to a normal standard chow diet (No. 322-7-1; Superfeed Co., Wonju, Republic of Korea) and tap water. Throughout the experiment, the animals were housed, four or five per cage, in laminar flow cages maintained at a temperature of 22 ± 2°C, and relative humidity of 50–60%, with a 12:12 h light-dark cycle. The rats were fasted for at least 24 h prior to start the experiments. Each animal was anaesthetized with ether and the right femoral vein (for i.v. DOX) and right femoral vein artery (for blood sampling) were cannulated with a polyethylene tube (SP45; i.d., 0.58 mm, o.d., 0.96 mm; Natsume Seisakusho Co. Ltd, Tokyo, Japan).

Rats were randomly divided into two groups (n = 6, each); oral group [50 mg (5 ml)/kg of DOX dissolved in a distilled water] without (control) or with 0.5, 3 and 10 mg/kg of oral quercetin (suspended in distilled water; total oral volume of 3.0 ml/kg), and intravenous group (10 mg/kg of DOX dissolved in 0.9% NaCl solution; total injection volume of 1.5 ml/kg). A feeding tube was used for oral administration of DOX and quercetin. Quercetin was administered 30 min prior to oral or i.v. administration of DOX. A blood sample (0.45 ml) was collected into heparinized tubes via the femoral artery at 0 (control), 0.017 (end of infusion), 0.1, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12 and 24 h for intravenous study, and 0, 0.1, 0.25, 0.5, 1, 2, 3, 5, 6, 8, 12 and 24 h for oral study. A whole

blood (approximately 1 ml) collected from untreated rats was infused via the femoral artery at 0.25, 1, 3, 8 and 12 h, respectively, to replace blood-loss due to blood sampling. The blood samples were centrifuged (13,000 rpm, 5 min), and a 200-μl aliquot of plasma samples was stored at –40˚C until the HPLC analysis.

2.3. HPLC analysis of DOX

The concentration of DOX in the sample were analysed by a slight modification of a reported HPLC method (Andersen *et al*. 1993). Briefly, a 50-μl aliquot of daunorubicin (1 μg/ml; internal standard), and a 1-ml aliquot of acetonitrile were added to a 200-μl aliquot of sample to deproteinize. The mixture was then stirred for 2 min and centrifuged (13,000 rpm, 10 min). A 0.8-ml aliquot of the upper layer was transferred to another clean tube, and then evaporated under a gentle stream of nitrogen gas at 38˚C. The residue was reconstituted in 200 μl mobile phase prior to injection (50 μl) into a C_{18} reverse phase column (ODS ThermoHypersil; 4.6 mm, i.d. × 150 mm; 5.0 μm; Thermo Electron Co., MA, USA). The mobile phase consisted of 20 mM phosphate buffer (pH 3.8):acetonitrile:methanol (45:20:35; v/v/v). The flow-rate of the mobile phase was 1.0 ml/min and the column eluent was monitored using a fluorosence detector at an excitation wavelength of 460 nm and an emission cut-off filter of 580 nm. The retention times of DOX and dounorubicin (internal standard) were approximately 3.5 and 5.8 min, respectively (Figure 4). The detection limit of DOX in rat´s plasma was 2 ng/ml. The intra- and inter-day coefficients of variation of DOX were below 11.3% (Figure 4).

2.4. Pharmacokinetic analysis

The following pharmacokinetic parameters were calculated using the non-compartmental method (WinNonlin software version 4.1; Pharsight Corporation, Mountain View, CA, USA); the total area under the plasma concentration–time curve from time zero to time infinity (AUC), the total body clearance (CL), the volume of distribution at steady state (Vss), and the terminal half-life ($t_{1/2}$), and the extent of absolute oral bioavailability (F). The peak plasma concentration (C_{max}) and time to reach C_{max} (T_{max}) were directly read from the experimental data.

2.5. Statistical analysis

A p-value < 0.05 was deemed to be statistically significant using a Duncan's multiple range test of Statistical Package of Social Sciences (SPSS) *posteriori* analysis of variance (ANOVA) program among the three means for the unpaired data. All data are expressed as mean ± standard deviation.

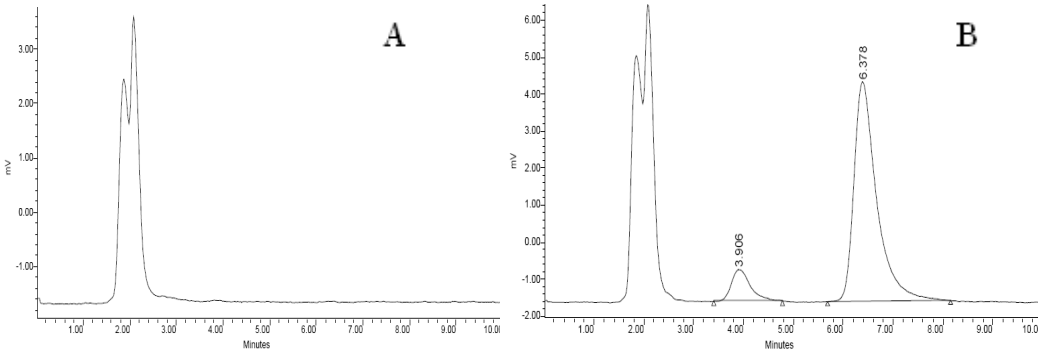

Figure 3. Typical HPLC chromatograms of the rat's blank plasma (A) and the plasma spiked with DOX (3.9 min) and daunorubicin (internal standard; 6.4 min) (B).

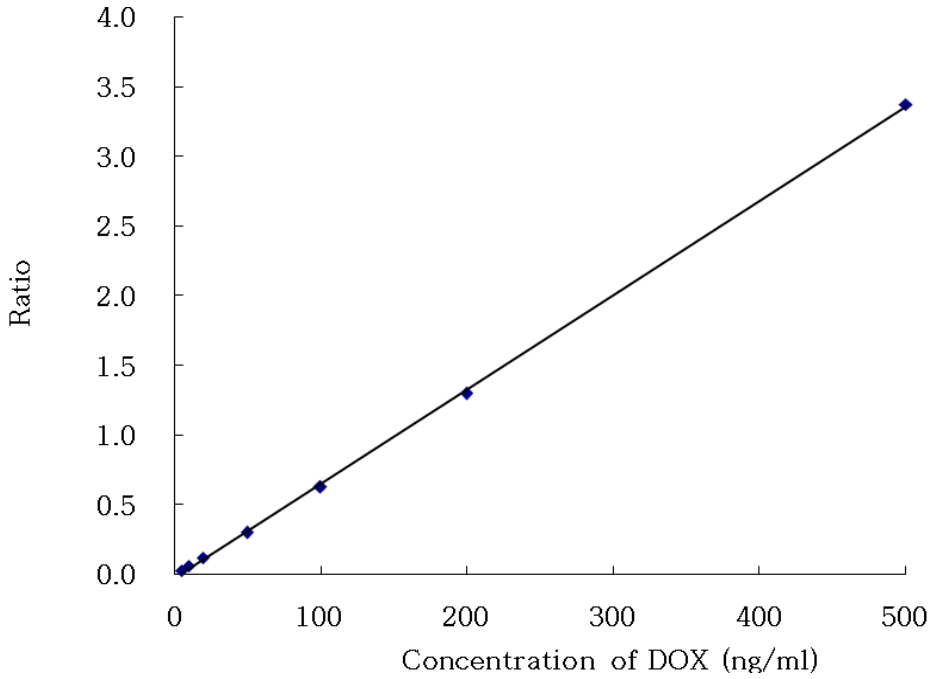

Figure 4. Typical calibration curve of DOX when spiked into the rat's blank plasma. The typical equation describing the calibration curve in rat's plasma was $y = 0.0068\ x - 0.021$, where y is the peak area ratio of DOX to daunorubicin and x is the concentration of DOX.

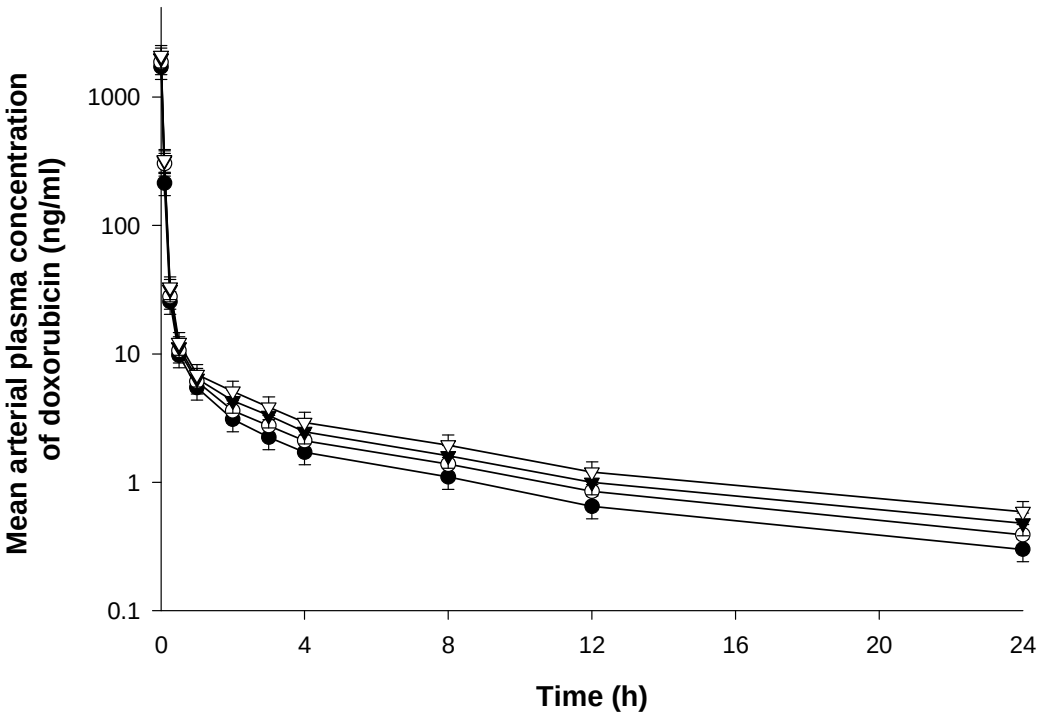

Figure 5. Mean arterial plasma concentration–time profiles of i.v. DOX (10 mg/kg) without (●) or with 0.5 mg/kg (○), 3 mg/kg (▼) or 10 mg/kg (▽) of quercetin to rats (*n* = 6, each). Bars represent the standard deviation.

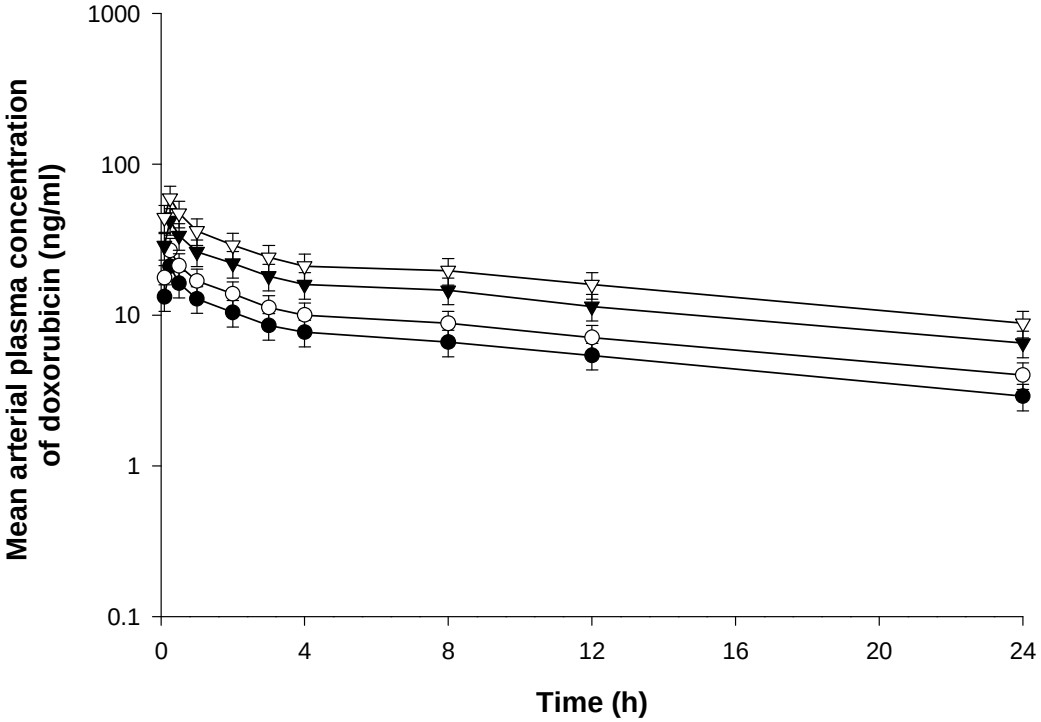

Figure 6. Mean arterial plasma concentration–time profiles of oral DOX (50 mg/kg) without (●) or with 0.5 mg/kg (○), 3 mg/kg (▼) or 10 mg/kg (▽) of quercetin to rats (*n* = 6, each). Bars represent the standard deviation.

Table 1. Mean (± S.D.) pharmacokinetic parameters of i.v. DOX (10 mg/kg) with or without of oral quercetin to rats (n = 6, each).

Parameters	DOX (Control)	DOX + Quercetin		
		0.5 mg/kg	3 mg/kg	10 mg/kg
AUC (ng·h/ml)[a]	1200 ± 142	1430 ± 267	1530 ± 249	1620 ± 424
Terminal half-life (h)	8.90 ± 2.32	8.88 ± 1.51	9.79 ± 1.91	9.98 ± 2.64
CL (l/h/kg)[b]	42.2 ± 5.07	35.9 ± 6.88	33.5 ± 5.48	32.7 ± 8.35
Vss (l/kg)	120 ± 57.5	102 ± 33.3	114 ± 48.2	119 ± 35.0
MRT (h)	2.87 ± 1.45	2.90 ± 1.11	3.42 ± 1.53	3.74 ± 1.23

[a] Control was significantly ($p < 0.05$) different from DOX + Quercetin 10 mg/kg.

[b] Control was significantly ($p < 0.05$) different from DOX + Quercetin 3 and 10 mg/kg.

Table 2. Mean (± S.D.) pharmacokinetic parameters of oral DOX (50 mg/kg) with or without of oral quercetin to rats (n = 6, each).

Parameters	DOX (Control)	DOX + Quercetin		
		0.5 mg/kg	3 mg/kg	10 mg/kg
AUC (ng·h/ml)[a]	210 ± 105	322 ± 115	499 ± 201	691 ± 132
Terminal half-life (h)	14.7 ± 7.12	20.1 ± 11.0	14.0 ± 3.37	18.8 ± 9.70
C_{max} (ng/ml)[a]	21.3 ± 8.94	27.3 ± 8.28	46.9 ± 9.80	68.3 ± 8.88
T_{max} (h)	0.25 (0.25–0.5)	0.25 (0.25–0.5)	0.25 (0.25–0.5)	0.25 (0.25)
F (%)	17.5	22.5	32.6	42.7

[a] Control and DOX + Quercetin 0.5 mg/kg were significantly ($p < 0.05$) different from DOX + Quercetin 3 mg/kg, and 10 mg/kg.

3. Results

The mean arterial plasma concentration–time profiles of i.v. DOX in the presence or absence of oral quercetin are shown in Fig. 5. The relevant pharmacokinetic parameters are listed in Table 1. In the presence of quercetin, the AUC of i.v. DOX was significantly ($p < 0.05$) greater (35.0% increase for 10 mg/kg of quercetin) than that without quercetin. The CL of i.v. DOX was significantly ($p < 0.05$) decreased in the presence of quercetin (20.6% and 22.5% decrease for 3 and 10mg/kg of quercetin, respectively) than that without quercetin. Other pharmacokinetic parameters of i.v. DOX listed in Table 1 were not significantly different by quercetin.

The mean arterial plasma concentration–time profiles of oral DOX in the presence or absence of oral quercetin are shown in Fig. 6. The relevant pharmacokinetic parameters are listed in Table 2. In the presence of quercetin, the AUC of oral DOX was significantly ($p < 0.05$) greater (138% and 229% increase for 3 and 10mg/kg of quercetin, respectively) than that without quercetin. The C_{max} of DOX was significantly ($p < 0.05$) higher in the presence of quercetin (120% and 221% increase for 3 and 10 mg/kg of quercetin, respectively) than that without quercetin. The F in the presence of quercetin was considerably greater (28.6%, 86.3% and 144% increase for 0.5, 3 and 10 mg/kg of quercetin, respectively) than that without quercetin. Other pharmacokinetic parameters of DOX listed in Table 2 were not significantly different by quercetin.

4. Discussion

With the great interest in herbal products as alternative medicines, much effort is currently being expanded toward identifying natural compounds from plant origins that modulate P-gp as well as metabolic enzymes. However, there is far less information on the pharmacokinetic interactions between herbal products and anticancer agents. Therefore, more preclinical and clinical investigations on the herbal constituents–drug interaction should be performed to prevent potential adverse reactions or to utilize those interactions for therapeutic benefits. Therefore, the present thesis evaluated the effects of oral quercetin, a naturally occurring flavonoid, on the bioavailability of DOX in rats, to examine possible drug interactions between quercetin and DOX via the dual inhibition of CYP3A and P-gp by quercetin.

Based on the broad overlap in the substrate specificities as well as co-localization in the small intestine, the primary site of absorption for orally administered drugs, CYP3A4 and P-gp have been recognized as a concerted barrier to the drug absorption (Cummins *et al*., 2002; Benet *et al*., 2003). Therefore, dual inhibitors against both CYP3A4 and P-gp should have a great impact on the bioavailability of many drugs.

CYP3A subfamily and P-gp inhibitors might interact with DOX and contribute to the substantial alteration of their pharmacokinetic parameters. Dupuy *et al*. (2003) reported that the AUC and plasma concentrations of moxidectin (a substrate for P-gp and CYP3A subfamily) increased when used concomitantly with 10 mg/kg of quercetin in lambs. It is possible that the concomitant administration of quercetin might affect the bioavailability or pharmacokinetics of oral DOX.

As summarized in Table 1, the presence of quercetin had no effect on pharmacokinetic parameters of i.v. DOX, although AUC of i.v. DOX increased with increasing oral quercetin doses. This suggests that the inhibition of metabolism of DOX via CYP3A subfamily is not considerable.

As listed in Table 2, the presence of quercetin significantly increased the AUC of oral DOX. These results were consistant with the report by Dupuy *et al*. (2003) suggested that the presence of quercetin might inhibit the CYP3A and the P-gp pathway because orally administered DOX is a substrate for CYP3A-catalyzed metabolism and P-gp-mediated

efflux in the intestine and liver (Guengerich and Kim, 1990; Miniscalco *et al*., 1992; Scambia *et al*., 1994; van Cooray *et al*., 2004 and Zanden *et al*., 2005). Quercetin might be able to improve the oral bioavailability of DOX by altering its absorption pattern or reducing the gut wall metabolism of this drug. These results were consistent with the report by Choi *et al*. (2004) in that the presence of quercetin significantly increased the AUC and C_{max} of paclitaxel, a P-gp and CYP3A4 substrate, in rats, and the report by Shin *et al*. (2006) in that quercetin significantly increased the AUC of tamoxifen in rats.

Furthermore, since the present study raised the awareness about the potential drug interactions by concomitant use of quercetin with DOX, the dosage regimen of DOX should be taken into consideration, if this result is confirmed in clinical study.

5. Conclusion

The enhanced bioavailability of i.v. DOX by oral flavonoids may be due to the inhibition of CYP3A subfamily in the liver by flavonoids. The enhanced bioavailability of oral DOX by oral quercetin may be due to the inhibition of both P-gp and CYP3A subfamily in the intestine and/or liver.

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Part II. Effect of Morin on the Bioavailability of Doxorubicin in Rats

Abstract

Doxorubicin (DOX), an anthracycline antibiotic, possesses broad-spectrum antineoplastic activity, and is one of the most important anticancer agents. The purpose of this thesis is to investigate the effects of morin, the antioxidant, on the bioavailability or pharmacokinetics of DOX in rats. Thus, DOX was administered intravenously (i.v.; 10 mg/kg) or orally (p.o.; 50 mg/kg) with or without oral morin (0.5, 3 and 10 mg/kg).

In the presence of morin, the total area under the plasma concentration–time curve from time zero to time infinity (AUC) of DOX was significantly greater than that of the control. In the presence of 3 and 10 mg/kg of morin, the peak concentration (C_{max}) was significantly higher than that of the control. Consequently, the absolute bioavailability (AB) of DOX in the presence of morin was 3.7–8.3%, which was significantly enhanced compared with that of the control group (2.9%). The relative bioavailability (RB) of DOX was 1.30 to 2.90 times higher than that of the control group. Compared to the intravenous control, the presence of 10 mg/kg of oral morin significantly increased the i.v. AUC of DOX, but other pharmacokinetic parameters were not significantly affected. The enhanced bioavailability of oral DOX by oral morin may be due to the inhibition of both P-glycoprotein (P-gp) and cytochrome P450 (CYP) 3A subfamily in the intestine and/or liver by morin.

This result may suggest that the development of oral DOX combination with morin is feasible, which is more convenient than the i.v. dosage forms. Furthermore, since the present study raised the awareness about the potential drug interactions by concomitant use of DOX with morin, the dosage regimen of DOX should be taken into consideration, if this result is confirmed in clinical studies.

Key words: Bioavailability, Doxorubicin, Morin, P-gp, CYP3A subfamily, Rats

1. Introduction

Many researchers have attempted to circumvent inhibition of P-glycoprotein (P-gp) during cytotoxic drug administration. For example, P-gp inhibitors, such as verapamil, cyclosporine, valspodar, GF120918 or LY357739 have previously been used to enhance intracellular accumulation of drugs in the multidrug resistance (MDR) cells (Avendano *et al*., 2002; Gottesman *et al*., 2002). P-gp, an important member of the ATP binding cassette (ABC) family which effluxes substrates out of cells, is highly expressed in solid tumours of epithelial origin, such as the colon (Cordon-Cardo *et al*., 1990) kidney (Fojo *et al*., 1987) and breast (Merkel *et al*., 1989). Cytochrome P450 (CYP) 3A subfamily, a major phase I drug metabolizing enzyme, are co-localized with P-gp in the liver and intestine (Wang *et al*., 2001; Fakhoury *et al*., 2005). Thus, a combined role of P-gp and CYP3A subfamily could decrease oral bioavailability of drugs which are substrates of P-gp and CYP3A subfamily.

Doxorubicin (DOX), an anthracycline glycosidic anticancer drug, impairs DNA synthesis during tumor cell division. It is most commonly used for the treatment of lymphoma, osteosarcoma and other sarcomas, carcinomas, and melanoma (Schwarzbach *et al*., 2002; Langer *et al*., 2006; Lind *et al*., 2007; Lundberg *et al*., 2007; Smylie *et al*., 2007). DOX is a substrate of P-gp (Gustafson *et al*., 2005), and one or more enzymes of the CYP3A subfamily plays a role in DOX metabolism (Kivistö *et al*., 1995).

Flavonoids are the most abundant polyphenolic compounds present in the human diet, such as fruits, vegetables, tea, and red wine. Flavonoids have a variety of beneficial pharmacological properties, including antitumor, antioxidation, antiviral, and anti-inflammatory activities (Middleton *et al*., 2000). On the other hand, flavonoids are reported to modulate CYP3A4 and/or P-gp (Lee *et al*., 1994; Chieli *et al*., 1995; Di Pietro *et al*., 2002).

Morin (3, 5, 7, 2′, 4′-pentahydroxyflavone) is a flavonoid constituent of many herbs and fruits. *In vitro* studies morin have a variety of beneficial activities, including antioxidation (Kok *et al*., 2000), anti-mutagenesis (Francis *et al*., 1989) and anti-inflammation (Fang *et al*., 2003). Like its isomer quercetin, orally administered morin is absorbed easily in the intestine of rodents but it is mainly metabolized as glucuronides and sulfates (Hsin *et al*.,

2001; Hou *et al*., 2003). A previous study showed that morin inhibited P-gp mediated cellular efflux of P-gp substrates (Zhang and Morris, 2003). Buening *et al*. (1981) also reported that morin could inhibit cytochrome P-450 reductase in human liver microsomes. This implied that morin might affect the absorption, metabolism, and elimination of DOX. Morin significantly increased the area under the plasma concentration–time curve (AUC) of paclitaxel, etoposide and tamoxifen in rats, which might be due to the inhibition of P-gp efflux and CYP3A subfamily metablism in the intestine (Choi *et al*., 2006; Li *et al*., 2007; Shin *et al*., 2008). Furthermore, morin and anticancer agents could be prescribed concomitantly for improving cancer therapy, because the morin has beneficial effect such as anticancer and antioxidant activity.

Therefore, the aim of this study was to examine the bioavailability and pharmacokinetics of DOX after the oral or intravenous administration of DOX with morin in rats.

2. Materials and methods

2.1. Chemicals

DOX were obtained from Boryung Pharmaceutical Co. (Seoul, Republic of Korea). Morin and dounorubicin [internal standard for the high-performance liquid chromatographic (HPLC) analysis of DOX] were purchased from Sigma–Aldrich Co. (St. Louis, MO). Other chemicals were of reagent or HPLC grade.

2.2. Animals

Male Sprague-Dawley rats, 7–8 weeks old and weighing 270–300 g, purchased from the Dae Han Laboratory Animal Research Company (EumSung, Republic of Korea) were given free access to a commercial rat chow diet (No. 322-7-1; Superfeed Company, Wonju, Republic of Korea) and tap water. They were maintained in a clean room (College of Pharmacy, Chosun University) at a temperature of 22 ± 2°C with 12-h light and dark cycles and a relative humidity of 50–60%. The rats were acclimated under these conditions for at least 1 week. The all protocol of this animal study was approved by Animal Care Committee of the Chosun University (Gwangju, Republic of Korea). Each rat was fasted for at least 24 h prior to start of the experiment. The left femoral artery (for blood sampling) and the left femoral vein (for drug administration only for intravenous study) were cannulated using a polyethylene tube (SP45; i.d., 0.58 mm, o.d., 0.96 mm; Natsume Seisakusho Co. Ltd, Tokyo, Japan) while each rat was under light ether anesthesia.

2.3. Intravenous and oral administration of DOX

Rats were randomly divided into two groups (n = 6, each); oral group [50 mg (5 ml)/kg of DOX dissolved in a distilled water] without (control) or with 0.5, 3 and 10 mg/kg of oral morin (mixed in distilled water; total oral volume of 3.0 ml/kg), and intravenous group (10 mg/kg of DOX dissolved in 0.9% NaCl solution; total injection volume of 1.5 ml/kg). A feeding tube was used for oral administration of DOX and morin. Morin was administered 30 min prior to oral administration of DOX. A blood sample (0.45 ml) was collected into heparinized tubes via the femoral artery at 0 (control), 0.017 (end of

infusion), 0.1, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12 and 24 h for intravenous study, and 0, 0.1, 0.25, 0.5, 1, 2, 3, 5, 6, 8, 12 and 24 h for oral study. A whole blood (approximately 1 ml) collected from untreated rats was infused via the femoral artery at 0.25, 1, 3, 8 and 12 h, respectively, to replace blood-loss due to blood sampling. The blood samples were centrifuged (13,000 rpm, 5 min), and a 200 μl aliquot of plasma samples was stored at –40˚C until the HPLC analysis.

2.4. HPLC analysis of DOX

The HPLC assay of Andersen *et al*. (1993) was used to analyze DOX levels, with minor modifications. Briefly, a 50 μl aliquot of daunorubicin (1 μg/ml; internal standard), and a 1 mL aliquot of acetonitrile was added to each 200 μl sample to precipitate proteins and extract DOX. The mixture was then stirred for 2 min and centrifuged (13,000 rpm, 10 min). A 0.8 mL aliquot of the upper layer was transferred to another clean microtube, and then evaporated under a gentle stream of nitrogen gas at 38˚C. The residue was reconstituted in 200 μl mobile phase prior to injection (50 μl) into a C_{18} reverse phase column (ODS ThermoHypersil; 4.6 mm, i.d.×150 mm; 5.0 μm; Thermo Electron Co., MA, USA). The mobile phase consisted of 20 mM phosphate buffer (pH 3.8):acetonitrile:methanol (45:20:35; v/v/v). The flow-rate of the mobile phase was 1.0 ml/min and the column eluent was monitored using a fluorosence detector at an excitation wavelength of 460 nm with an emission cut-off filter of 580 nm. The retention times of DOX and dounorubicin (an internal standard) were approximately 3.5 and 5.8 min, respectively. The detection limit of DOX in rat's plasma was 2 ng/ml. The intra- and inter-day coefficients of variation of DOX were below 11.3%.

2.5. Pharmacokinetic analysis

The following pharmacokinetic data were analyzed using the non-compartmental method (WinNonlin software version 4.1; Pharsight Corporation, Mountain View, CA, USA). The half-life ($t_{1/2}$) was calculated by $0.693/K_{el}$. The peak concentration (C_{max}) and the time to reach peak concentration (T_{max}) of DOX directly read from the experimental data. The area under the plasma concentration–time curve (AUC_{0-t}) from time zero to the time of last measured concentration (C_{last}) was calculated by the linear trapezoidal rule. The AUC zero

to infinite ($AUC_{0-\infty}$) was obtained by the addition of AUC_{0-t} and the extrapolated area was determined by C_{last}/K_{el}. The total body clearance for intravenous (CL_t) and oral administration (CL/F) was calculated from the quotient of the dose (D) and $AUC_{0-\infty}$. The absolute bioavailability (AB) was calculated by $AUC_{oral}/AUC_{IV} \times Dose_{IV}/Dose_{oral} \times 100$, and the relative bioavailability (RB) was estimated by $AUC_{with\ morin}/AUC_{control} \times 100$.

2.6 Statistical analysis

A *p*-value < 0.05 was deemed to be statistically significant using a Duncan's multiple range test of Statistical Package of Social Sciences (SPSS) *posteriori* analysis of variance (ANOVA) program among the three means for the unpaired data. All data are expressed as mean ± standard deviation.

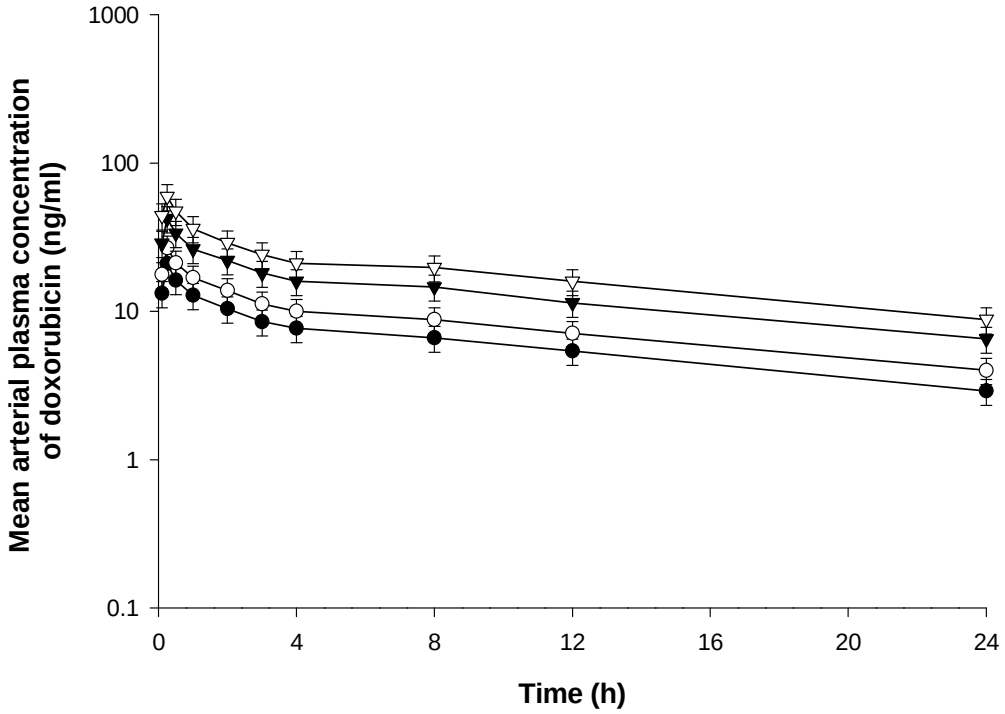

Figure 7. Mean arterial plasma concentration–time profiles of DOX after oral administration of DOX at a dose of 50 mg/kg in the presence of morin at doses of 0.5 mg/kg (○; *n* = 6), 3 mg/kg (▼; *n* = 6) and 10 mg/kg (▽; *n* = 6) or absence (●; *n* = 6) of morin to rats. Bars represent stnandard deviation.

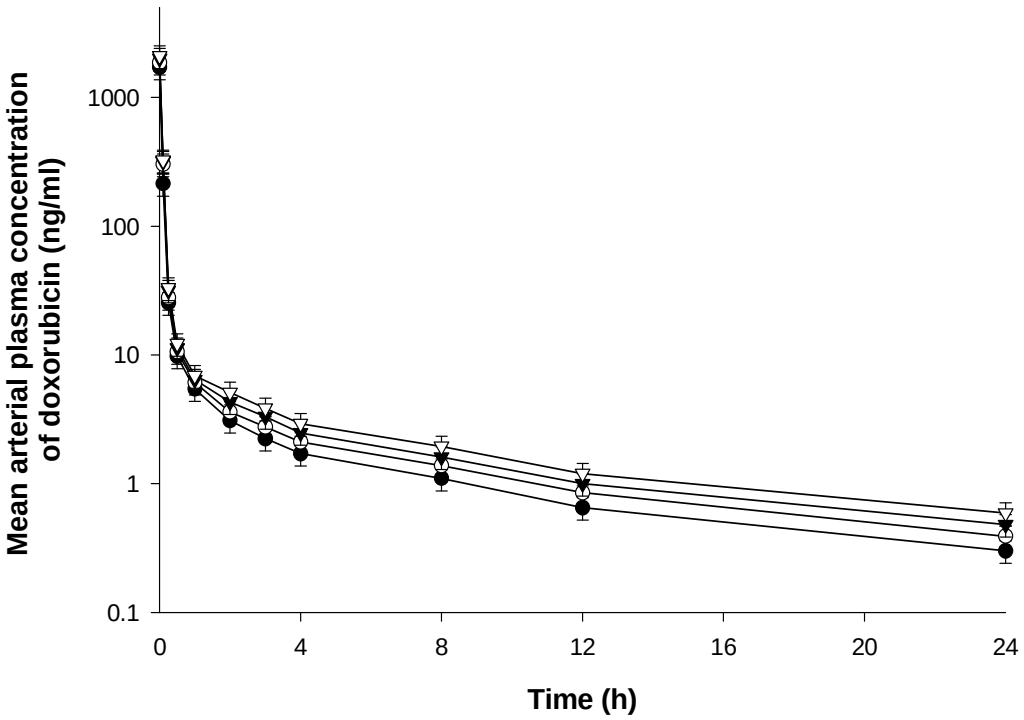

Figure 8. Mean arterial plasma concentration–time profiles of DOX after intravenous administration of DOX at a dose of 10 mg/kg in the presence of morin at doses of 0.5 mg/kg (○; *n* = 6), 3 mg/kg (▼; *n* = 6) and 10 mg/kg (▽; *n* = 6) or absence (●; *n* = 6) of morin to rats. Bars represent stnandard deviation.

Table 3

Mean (± S.D.) pharmacokinetic parameters of DOX after oral administration of DOX at a dose of 50 mg/kg in the presence or absence (control) of morin at doses of 0.5, 3 and 10 mg/kg to rats (n = 6, each)

Parameters	DOX (Control)	DOX + Morin		
		0.5 mg/kg	3 mg/kg	10 mg/kg
AUC (ng·h/ml)	214 ± 41.1	279 ± 57.2*	453 ± 94.6**	620 ± 127**
C_{max} (ng/ml)	21.3 ± 4.30	26.8 ± 5.60	42.2 ± 8.82*	59.4 ± 12.3**
T_{max} (h)	0.25	0.25	0.25	0.25
CL/F (l/min/kg)	4.66 ± 0.98	3.58 ± 0.75	2.21 ± 0.46*	1.61 ± 0.34*
$t_{1/2}$ (h)	13.8 ± 2.90	14.8 ± 3.11	14.9 ± 3.12	15.2 ± 3.17
AB (%)	2.9 ± 0.55	3.7 ± 0.74*	6.1 ± 1.21**	8.3 ± 1.70**
RB (%)	100	130	212	290

*$p < 0.05$, **$p < 0.01$ significant difference compared with the control.

AUC: area under the plasma concentration–time curve from zero to time infinity; C_{max}: peak concentration; T_{max}: time to reach peak concentration; CL/F: total body clearance; $t_{1/2}$: the terminal half-life; AB: absolute bioavailability; RB: relative bioavailability.

Table 4

Mean (± S.D.) pharmacokinetic parameters of DOX after intravenous administration of DOX at a dose of 10 mg/kg in the presence or absence (control) of morin at doses of 0.5, 3 and 10 mg/kg to rats (n = 6, each)

Parameters	DOX (Control)	DOX + Morin		
		0.5 mg/kg	3 mg/kg	10 mg/kg
AUC (ng·h/ml)	1500 ± 312	1765 ± 367	1878 ± 387	1992 ± 406*
CL_t (ml/min/kg)	111 ± 25.2	94.5 ± 19.6	88.7 ± 14.4	83.7 ± 13.7
$t_{1/2}$ (h)	8.05 ± 1.70	8.37 ± 1.73	8.61 ± 1.80	8.83 ± 1.82

*$p < 0.05$, significant difference compared with the control.

AUC: area under the plasma concentration–time curve from zero to infinity; CL_t: total body clearance; $t_{1/2}$: terminal half-life.

3. Results

The mean plasma concentration–time profiles of DOX following oral administration to rats in the presence or absence of oral morin are illustrated in Figure 7. The mean pharmacokinetic parameters of DOX are also listed in Table 3. As shown in Table 3, the presence of morin significantly altered the pharmacokinetic parameters of DOX. Compared with the control group (given oral DOX alone), the presence of morin significantly increased the area under the plasma concentration time curve from zero to time infinity (AUC) ($p < 0.05$ at 0.5 mg/kg; $p < 0.01$ at 3 and10 mg/kg) and the peak concentration (C_{max}) ($p < 0.05$ at 3 mg/kg; $p < 0.01$ at 10 mg/kg) of DOX by 30.4–190% and 98.1–179%, respectively, and significantly reduced the total body clearance (CL/F) of DOX ($p < 0.05$, 3 and 10 mg/kg) by 52.6–65.4%. The absolute bioavailability (AB) of DOX was significantly elevated ($p < 0.05$ at 0.5 mg/kg; $p < 0.01$ at 3 and 10 mg/kg) by 3.7–8.3%, compared with the control group (2.9%). The relative bioavailability (RB) of DOX in the presence of morin (0.5, 3 and 10 mg/kg) was 1.30 to 2.90 times higher. There was no significant difference in the time to reach peak concentration (T_{max}) or the terminal half-life ($t_{1/2}$) of DOX in the presence of morin.

The mean plasma concentration–time profiles of DOX following intravenous administration to rats in the presence or absence of oral morin are illustrated in Figure 8. The mean pharmacokinetic parameters of DOX are also listed in Table 4. Table 4 shows the corresponding pharmacokinetic parameters. Compared with the control group, the presence of morin at 10 mg/kg increased the AUC (32.8%) of i.v. DOX significantly ($p < 0.05$). The C_{max}, $t_{1/2}$ and T_{max} of i.v. DOX were not affected by morin.

4. Discussion

With the great interest in herbal products as alternative medicines, much effort is currently being expanded toward identifying natural compounds from plant origins that modulate P-gp as well as metabolic enzymes. However, there is far less information on the pharmacokinetic interactions between herbal products and anticancer agents. Therefore, more preclinical and clinical investigations on the herbal constituents–drug interaction should be performed to prevent potential adverse reactions or to utilize those interactions for therapeutic benefits. Therefore, the present thesis evaluated the effects of morin, a naturally occurring flavonoid, on the bioavailability of DOX in rats, to examine possible drug interactions between morin and DOX via the dual inhibition of CYP3A subfamily and P-gp by morin.

Based on the broad overlap in the substrate specificities as well as co-localization in the small intestine, the primary site of absorption for orally administered drugs, CYP3A and P-gp have been recognized as a concerted barrier to the drug absorption (Cummins *et al*., 2002; Benet *et al*., 2003). Therefore, dual inhibitors against both CYP3A and P-gp should have a great impact on the bioavailability of many drugs.

CYP3A subfamily and P-gp inhibitors might interact with DOX and contribute to the substantial alteration of their pharmacokinetic parameters. Since cyclosporin and verapamil, both substrates for CYP3A, increased DOX plasma concentrations, it is possible that one or more enzymes of the CYP3A subfamily play a role in DOX metabolism (Kivistö *et al*., 1995). Morin inhibited P-gp-mediated efflux of daunomycin, which was comparable to verapamil, a potent P-gp inhibitor (Buening *et al*., 1981; Zhang and Morris, 2003). It is possible that the concomitant administration of morin might affect the bioavailability or pharmacokinetics of orally administered DOX.

As listed in Table 3, the presence of morin significantly increased the AUC and reduced the CL/F of oral DOX. These results were consistant with the report by Buening *et al*. (1981) and Zhang and Morris, (2003) suggested that the presence of morin might inhibit the CYP3A and the P-gp pathway because orally administered DOX is a substrate P-gp-mediated efflux and metabolited by CYP3A subfamily in the intestine and/or liver. Morin might be able to improve the oral bioavailability of DOX by altering its absorption pattern

or reducing the gut wall metabolism of this drug. These results were consistent with the report by Choi *et al*. (2006) in that the presence of morin significantly increased the AUC and C_{max} of paclitaxel, a P-gp and CYP3A4 substrate, in rats, and the report by Li *et al*. (2007) in that morin significantly increased the AUC of etoposide in rats. Shin *et al*. (2008) also reported that the presence of the morin at doses of 2.5 and 7.5 mg/kg significantly increased the AUC and C_{max} of tamoxifen, a P-gp and CYP3A4 substrate, in rats.

As listed in Table 4, the presence of 10 mg/kg of morin significantly increased the AUC of intravenous DOX. However, morin had no effect on other pharmacokinetic parameters of intravenous DOX, although it exhibited a significant effect on the bioavailability of oral DOX. This result is contrast to a report by Li *et al*. (2007) showing that the presence of morin did not increase the AUC of intravenous etoposide in rats.

Collectively, the bioavailability of oral DOX was significantly increased by the concomitant use of morin via the inhibition of the P-gp mediated efflux and first-pass metabolism of DOX in the intestine and/or liver. This result may suggest that the development of oral DOX preparations as a combination with morin is feasible, which is more convenient than the i.v. dosage forms.

Furthermore, since the present study raised the awareness about the potential drug interactions by concomitant use of morin with DOX, the dosage regimen of DOX should be taken into consideration, if this result will be confirmed in clinical study.

5. Conclusion

The presence of morin enhanced the oral bioavailability of DOX, which might be attributed to the promotion of intestinal absorption and a reduction of the first-pass metabolism of DOX. This result may suggest that the development of oral DOX preparations as a combination with morin is feasible, which is more convenient than the i.v. dosage forms.

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저작물 이용 허락서

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논문제목	한글: 흰쥐에서 퀠세틴과 모린이 독소루비신의 생체이용율에 미치는 영향. 영문: Effects of quercetin and morin on the bioavailability of doxorubicin in rats				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 –

1. 저작물의 DB 구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함.
2. 위의 목적을 위하여 필요한 범위 내에서의 편집・형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포・전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5 년으로 하고, 기간종료 3 개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1 개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음.
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송・출력을 허락함.

동의여부 : 동의 (√) 반대 ( )

2009 년 02 월

저작자: 박영길 (서명 또는 인)

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