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The possibility of low isomerization of β-lapachone in the human body

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ABSTRACT

β-Lapachone has been reported to have anticancer and various other therapeutic effects, but is limited in clinical applications by its low bioavailability. pH-Dependent isomerization can be suggested as one plausible factor influencing its low bioavailability. Since it is known that β-lapachone is converted to its isomer, α-lapachone in hydrochloric acid (HCl) solution, isomerization in the human body may be driven by HCl in the gastric fluid. The purpose of this study was to evaluate the possibility of isomerization of β-lapachone in the human body. Chemical reactions were conducted using simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 7.5) at 37°C. β-Lapachone was observed in SGF at 37°C for 1 hour and SIF for 3 hours. In addition, biofluid analysis was performed on plasma samples 1 hour and 4 hours, and on urine sample 12 hours after oral administration of 100 mg MB12066, a synthetic β-lapachone, in healthy adult male. All samples were analyzed using liquid chromatography-tandem mass spectrometry. Only β-lapachone peaks existed in the spectra obtained from SGF and SIF. No isomerization of β-lapachone was observed in the analysis of any of the human samples. In the current study, the possibility of pH-dependent isomerization of β-lapachone in the human body was not confirmed.

Keywords: Beta-Lapachone; Bioavailability; Liquid Chromatography; Tandem Mass Spectrometry; MB12066

INTRODUCTION

β-Lapachone (3,4-dihydro-2,2-dimethyl-2H-naphthol[1,2-b]pyran-5,6-dione, **Fig. 1**), an ortho-naphthoquinone naturally originating from the bark of the lapacho tree (*Tabebuia avellanedae*) [1], has been shown to have many beneficial effects, including anticancer [2,3], anti-inflammatory [4,5], anti-bacterial [6], and anti-aging properties [7]. β-Lapachone has also demonstrated therapeutic effects in various diseases, such as metabolic syndrome [8,9] and neurodegenerative disorders [10]. β-Lapachone is especially well known as an anticancer agent, affecting the activity of nicotinamide adenine dinucleotide (NAD) (P)H:quinone oxidoreductase 1 (NQO1). NQO1 is an antioxidant flavoenzyme involved in quinone reduction [11]. NQO1 is expressed in many tumors, including lung [12,13], breast [14], and pancreatic cancers [15]. The reduction of β-lapachone to hydroquinone occurs via NQO1 in the

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Conceptualization: Lee KM; Data curation:
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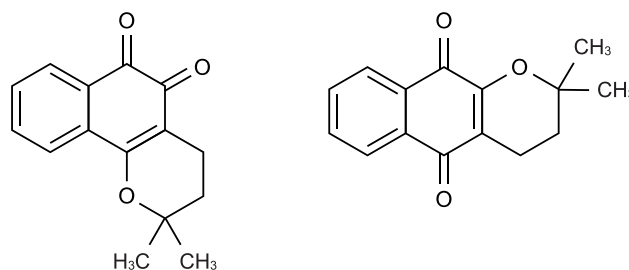
Figure 1. Structure of β-lapachone (left) and α-lapachone (right).

presence of NAD(P)H [16]. Since hydroquinone is unstable, it spontaneously reverts to stable β-lapachone through autoxidation, leading to a futile redox cycle [2,11]. This futile redox cycle causes the generation of intracellular reactive oxygen species, leading to the death of cancer cells [2,11,16].

Several clinical trials have been conducted to develop β-lapachone as a drug. ARQ 501 (ArQule, Burlington, MA, USA), a synthetic β-lapachone compound, was developed as an anticancer drug, and phase I and phase II clinical trials were conducted in adult patients [17,18].

MB12066 (KT&G Life Sciences, Suwon, Korea), another synthetic β-lapachone compound, was developed, and clinical trials were conducted in healthy Korean male adults to verify its therapeutic effects in metabolic syndrome, including obesity and alcoholic fatty liver disease [19,20]. β-Lapachone can play an important role in the treatment of metabolic syndrome through NQO1-mediated changes of the NAD^+/NADH ratio, which is important for energy metabolism in cells [8,21]. MB12066 was shown to induce NAD(P)H oxidation to stimulate adenosine monophosphate (AMP)-activated protein kinase (AMPK) signaling and mitochondrial function, thereby promoting glucose and lipid metabolism [8,21]. This can reduce free fatty acids, cholesterol, and glucose accumulation, which can increase energy consumption by promoting browning in white adipose tissue, and can also be effective in treating obesity and metabolic diseases such as fatty liver and dyslipidemia [8,9,21]. Although β-lapachone has various beneficial effects, it is limited in drug development by its low bioavailability [22].

Drug bioavailability refers to the rate and extent of a drug reaching systemic circulation after administration [23]. Poor bioavailability in the human body is known to be caused by low aqueous solubility and extensive first-pass metabolism. Drugs with poor aqueous solubility have low dissolution amounts and slow dissolution velocities [23]. In a preclinical pharmacokinetic study, the bioavailability of β-lapachone was measured as 15.54% when orally administered to rats [22]. Apart from β-lapachone [22,24,25], paclitaxel [26] and griseofulvin [27] are also examples of low water-soluble drugs. Orally administered drugs enter the liver through the portal circulation before reaching systemic circulation [23]; if a significant amount of a drug is metabolized by various metabolic enzymes due to extensive first-pass metabolism, bioavailability is lowered [23]. In addition to β-lapachone [22,28-32], lovastatin [33] and pentazocine [34] are examples of drugs that have low bioavailability due to extensive first-pass metabolism.

Another plausible cause for low bioavailability of β-lapachone is pH-dependent isomerization of β-lapachone in the human body. Isomerization is a reaction changing the chemical structure of a compound without changing its molecular composition. Isomerization is

one type of possible drug degradation reaction, rendering drugs inactive or unstable in gastrointestinal fluid [23,35]. Roxithromycin, an antibacterial agent, is one example of an agent with low bioavailability caused by pH-dependent isomerization [36]. Isomerization of roxithromycin in the gastric fluid to Z-roxithromycin affects the bioavailability of roxithromycin [36].

However, pH-dependent isomerization does not necessarily decrease bioavailability. Lycopene, a carotenoid pigment in tomatoes, is known to reduce the risk of prostate cancer [37]. Low pH in the stomach isomerizes lycopene to *cis*-lycopene, that is absorbed even more compared to the *trans* isomer, leading to increased bioavailability [38-40]. Accordingly, bioavailability may be increased or decreased depending on pH-dependent isomerization. It has long been known that β-lapachone is isomerized to α-lapachone in hydrochloric acid (HCl) solution [41-43]. α-Lapachone (**Fig. 1**), a para-naphthoquinone, has an anticancer effect by blocking deoxyribonucleic acid (DNA) topoisomerase II [44], although it has weaker pharmacological effect than β-lapachone [45,46]. In the stomach, HCl is secreted at a concentration of about 0.2–0.5% to aid in digestion. Therefore, some orally administered β-lapachone may be isomerized in the stomach by HCl. If this were to occur, the level of β-lapachone from the stomach to the small intestine would be reduced, and the amount of β-lapachone reaching the systemic circulation would be decreased, resulting in low bioavailability.

β-Lapachone may not be isomerized into α-lapachone because of low HCl concentration in the stomach. However, since there is no experiment to confirm isomerization of β-lapachone using biofluid samples, the purpose of this study was to evaluate the possibility of the isomerization of β-lapachone in the human body and to examine whether it could contribute to its low bioavailability. Two experiments were conducted: one in which reactions were performed in simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 7.5) at 37°C; and another in which plasma and urine samples of a healthy adult male were analyzed following oral administration of MB12066.

METHODS

Materials
β-Lapachone (MB12066) was obtained from KT&G Life Sciences. Co., Ltd.. α-Lapachone was purchased from BOC Sciences (Shirley, NY, USA). High-performance liquid chromatography (HPLC)-grade acetonitrile, formic acid (98–100% for analysis), ammonium acetate, and hydrochloric acid (HCl, 37%) were purchased from Merck (Darmstadt, Germany). Sodium chloride (NaCl), sodium hydroxide (NaOH) and potassium phosphate monobasic (KH_2PO_4) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized water was purified using a Milli-Q system (Merck Millipore, Burlington, MA, USA). Heparinized blank human plasma was purchased from Innovative Research, Inc. (Novi, MI, USA).

Methods
Preparation of β-lapachone standard solution, simulated gastric fluid (SGF), and simulated intestinal fluid (SIF)
β-Lapachone is sensitive to light, so the exposure of light was minimized during sample preparation and analysis [47]. A working standard solution of β-lapachone was prepared in acetonitrile at a concentration of 1 μg/mL. Then, 100 μL of β-lapachone solution was added

to 900 μL of SGF (pH 1.2) or SIF (pH 7.5) lacking enzyme, respectively. The process of making SGF and SIF was as follows [48]: SGF was made by adding 100 mg of NaCl and 0.35 mL of HCl to 49.65 mL of deionized water. SIF was made by dissolving 340 mg of KH_2PO_4 into 12.5 mL of deionized water. Then, 9.5 mL of 0.2 M NaOH and 20 mL of deionized water were added to the solution. To adjust the pH to 7.5, 0.2 M NaOH was used. Deionized water was added to bring the volume of the resulting solution to 50 mL. β-Lapachone in SGF or SIF were incubated in the water bath at 37°C for 1 hour or 3 hours, respectively. Each 100 μL sample was transferred to a separate tube.

Human plasma and urine samples preparation
Human plasma and urine samples from one of the subjects who participated in a pharmacokinetic study conducted in healthy Korean male adults following oral administration of a single 100 mg dose of MB12066 (ClinicalTrials.gov Identifier: NCT02338856) were used for analysis. The study protocol was approved by Institutional Review Board of Kyungpook National University Hospital, Daegu, Republic of Korea, and written informed consents were obtained from all subjects [19]. Plasma samples obtained at 1 hour and 4 hours after oral administration were used. Urine sample obtained from 0 to 12 hours after oral administration was used. Samples were stored at −70°C until analysis.

Plasma and urine samples were thawed at room temperature before preparation. Then, 100 μL of each sample was transferred to a microcentrifuge tube, and 200 μL of acetonitrile was added to each tube. The mixture was vortexed for 5 minutes, then centrifuged at 13,200 rpm for 10 minutes at 4°C. Each supernatant (100 μL) was transferred to a separate vial for analysis.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis
The reaction of β-lapachone in SGF and SIF was analyzed using an Alliance 2695 HPLC (Waters Corp., Milford, MA, USA) coupled with a Finnigan LXQ ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). HPLC was performed using a Zorbax Eclipse XDB-C18 column (2.1 × 150 mm, 3.5 μm, Agilent Technologies, Inc., Santa Clara, CA, USA). The column oven and autosampler temperature were set to 40°C and 20°C, respectively. An isocratic elution mode was used with 5 mM ammonium acetate and 0.1% formic acid in water (mobile phase A) and acetonitrile (mobile phase B) in a 50:50 (*v/v*) ratio. The flow rate was 0.2 mL/min, and the sample injection volume was 5 μL; each sample was analyzed over a 20 minutes run. Mass detection was performed in MS/MS positive electrospray ionization mode, with a scan range of 100–250 mass-to-charge ratio (*m/z*). The collision gas was helium, and the collision energy value was 20 eV. Other optimized MS parameters for MS/MS positive mode were as follows: capillary temperature, 320°C; capillary voltage, 32 V; flow rate of aux gas (N_2), 5 arbitrary units; flow rate of sheath gas (N_2), 20 arbitrary units; and tube lens voltage, 60 V.

For more accurate and precise analysis of human samples, an Agilent 1200 Series HPLC (Agilent) coupled with API 5000 mass spectrometer (SCIEX, Framingham, MA, USA) were used. The column and the column oven temperature were as above, as were the isocratic elution mode and the mobile phases. The autosampler temperature was set to 10°C. The sample injection volume was 2 μL, and each sample was analyzed over a 20 minutes run with a flow rate of 0.2 mL/min. Mass detection was performed in multiple reaction monitoring (MRM) positive electrospray ionization mode. The MRM transition (*m/z* 243 → 187) was monitored for more selective and sensitive analysis. The following optimized MS parameters for MRM positive mode were used: source temperature, 600°C; ion spray voltage, 5,500 V; declustering potential (DP), 46 V; entrance potential (EP), 8 V; collision energy (CE), 27 V; collision cell exit potential (CXP), 16 V.

RESULTS

The reaction to isomerization of β-lapachone in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF)

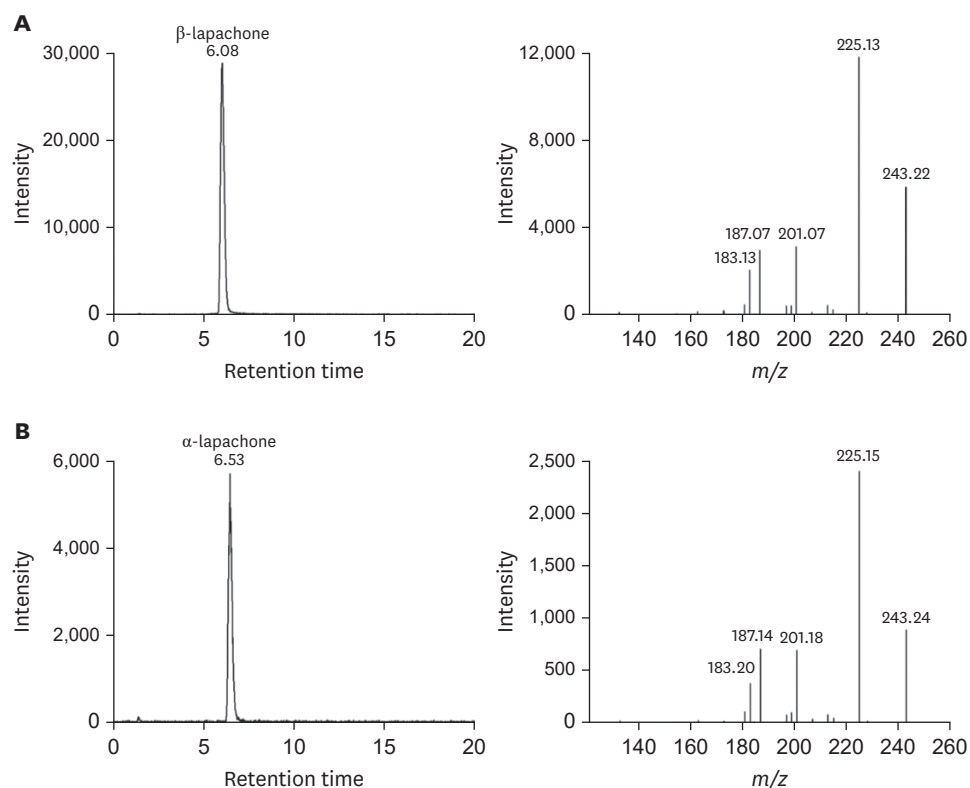Before analysis, peak chromatograms and MS/MS spectra of the β-lapachone standard solution (100 ng/mL) and α-lapachone standard solution (1,000 ng/mL) were identified (**Fig. 2**).

At a retention time of 6.08 minutes, β-lapachone was detected with a molecular ion ([M+H]$^+$) at m/z 243 (**Fig. 2**); at a retention time of 6.53 minutes, α-lapachone was detected with the same m/z value and MS/MS spectrum (**Fig. 2**). The ion at m/z 243 yielded fragments at m/z 225, 201, and 187.

Fig. 3 shows MS/MS chromatograms of β-lapachone in SGF and SIF, respectively. Based on the chromatogram peak of α-lapachone standard solution, there was no α-lapachone peak in SGF after 1 hour. Only β-lapachone peak was observed at a retention time of 6.09 minutes. Similarly, only β-lapachone existed in SIF after 3 hours. One peak at the retention time of 1.49 minutes was observed, but as shown in **Fig. 2**, this peak did not correspond to a α-lapachone peak.
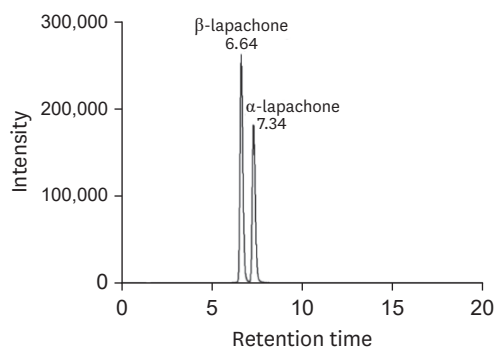
Human plasma and urine samples analysis

Fig. 4 shows MRM chromatograms of β-lapachone standard solution (100 ng/mL) and α-lapachone standard solution (200 ng/mL) in blank plasma. At a retention time of 6.64 minutes, β-lapachone was detected, and at a retention time of 7.34 minutes, α-lapachone was detected. α-Lapachone concentration was twice as high as β-lapachone, but the intensity was lower than β-lapachone.

Figure 2. Representative liquid chromatography-tandem mass spectrometry (LC-MS/MS) ion chromatograms and MS/MS spectra (scan range: mass to charge ratio (m/z) 100–250) of (A) β-lapachone standard solution (100 ng/mL) and (B) α-lapachone standard solution (1,000 ng/mL).
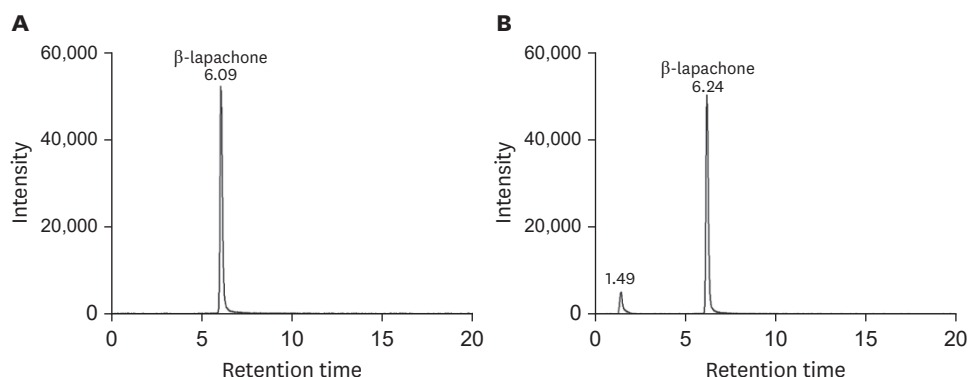
Figure 3. Representative liquid chromatography-tandem mass spectrometry ion chromatograms of (A) β-lapachone (100 ng/mL) in simulated gastric fluid (pH 1.2) and (B) in simulated intestinal fluid (pH 7.5).

Figure 4. Representative multiple reaction monitoring (mass to charge ratio [*m/z*] 243 → 187) chromatogram of β-lapachone standard solution (100 ng/mL) and α-lapachone standard solution (200 ng/mL) in blank plasma.

MRM chromatograms of β-lapachone in human plasma and urine samples are shown in **Fig. 5**, respectively. In plasma samples obtained 1 hour and 4 hours after oral administration of MB12066, (100 mg), based on the chromatogram peak of the α-lapachone standard solution in blank plasma, there were no α-lapachone peaks present in the two plasma samples (**Fig. 5A and B**). Similarly, in the urine sample obtained 0 to 12 hours after oral administration of MB12066, there was no α-lapachone peak observed (**Fig. 5C**). Several peaks before the retention time of 6.00 minutes were observed in human plasma and urine samples, but these peaks did not correspond to the peaks of the α-lapachone standard solution.
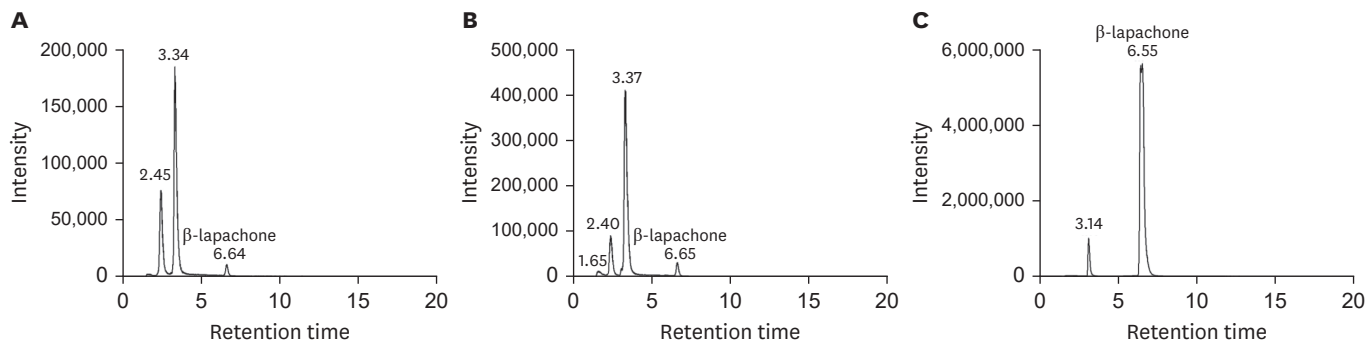
Figure 5. Representative multiple reaction monitoring (mass to charge ratio [*m/z*] 243 → 187) chromatograms of β-lapachone in human plasma samples obtained from (A) 1 hour and (B) 4 hours and human urine sample (C) obtained from 0 to 12 hours after oral administration of 100 mg MB12066.

DISCUSSION

Hooker [41] discovered that when β-lapachone was dissolved in HCl, α-lapachone was formed. Due to the continuous addition and removal of HCl to β-lapachone, the side ring of β-lapachone is opened and the β-lapachone structure is converted to α-lapachone [41]. Even though this process has been known for more than a century [41], this isomerization has not been well studied under acidic condition in the human body. In this study, we sought to observe isomerization of β-lapachone in the presence of HCl at concentrations between 0.2% and 0.5% through controlled reaction experiments, and also analyzed plasma and urine samples from a healthy Korean male who orally received MB12066. When making SGF, concentrated 37% HCl was used, accounting for 0.7% of the total SGF, leading to an actual HCl concentration in SGF of 0.259%, similar to the concentration of HCl in real gastric fluid. Liu *et al.* [25] conducted *in vitro* dissolution tests of β-lapachone at pH 1.4 for 1 hour and pH 6.5 for 3 hours. According to Wen *et al.* [49], the stomach transit time is 0.25 hours under fasting condition and 1 hour under fed condition, and the jejunum transit time is 1.7 hours regardless of food intake [49]. Accordingly, we conducted our experiments related to the pH-dependent isomerization of β-lapachone for 1 hour in SGF and 3 hours in SIF. Also, when analyzing human samples, we used plasma samples obtained 1 hour and 4 hours after oral administration of β-lapachone.

Our study did not identify isomerization of β-lapachone into α-lapachone in the human body. Delarmelina *et al.* [50] explained isomerization of β-lapachone and α-lapachone using the microsolvation model, and found that the isomerization of β-lapachone and α-lapachone varies depending on the concentration of HCl. The microsolvation model explains the energetics behind intermolecular interactions in the solvation process [51]. In HCl solution, HCl reacts with H_2O and donates H^+ to H_2O. Hydronium ions (H_3O^+) are formed by combining protons with water molecules, because hydrogen ions cannot exist stably in an aqueous solution. If the concentration of HCl is low, H^+ will not be released, resulting in fewer hydronium ions and more water molecules. Upon analysis of the microsolvation of the protonated sites and the solvation of the protonated sites with one more water molecule of β-lapachone and α-lapachone, the relative enthalpy values showed that β-lapachone is more stable than α-lapachone [50]. In addition, the isomerization of β-lapachone and α-lapachone can be related to diprotonated lapachol, but there is no sufficient proton donor in the low acid solution, resulting in the formation of monoprotonated lapachol [50]. When monoprotonated lapachol is formed, β-lapachone which is more stable than α-lapachone, is formed in a protonated state, because nucleophilic attack on the carbonyl group C_4=O occurs without an enthalpic barrier [50]. Delarmelina *et al.* [49] showed that more β-lapachone existed than α-lapachone in 9% HCl/acetic acid; the HCl concentration of SGF in our experiment was lower than 9%, so more β-lapachone would exist in that solution. In addition, we analyzed urine sample to identify the isomerization in the process of urine excretion following the metabolism of β-lapachone, but were unable to identify α-lapachone. Based on previous studies, β-lapachone is metabolized in the liver into various forms, but the metabolism of α-lapachone is not observed [28-30]. Lee *et al.* [29] explained that β-lapachone is metabolized by cytochrome P450 (CYP) to produce mono-oxygenated metabolites, while O-methylated metabolites are produced by membrane-bound catechol-O-methyltransferase (MB-COMT). Also, Cheng *et al.* [28] and Miao *et al.* [30] reported that the major metabolic pathway of β-lapachone was via glucuronidation, a phase II metabolic pathway, and several metabolites were produced by uridine diphosphate (UDP)-glucuronosyltransferase (UGT) using human liver S9 fractions and human hepatocytes, respectively.

There are several limitations to this study. First, SGF and SIF were used to make simulated gastrointestinal conditions, but only the pH of the stomach and the small intestine were applied, without any digestive enzymes. Second, since human plasma samples were taken in January 2015 [19], a lot of time passed prior to sample analysis. The stability of the plasma samples was not verified, and the β-lapachone peak after oral administration was low, even though the t_{max} of MB12066 is reported to be 3 hours [19]. Also, analyzing small number of human subject (n = 1) is a limitation in this study. After verifying the stability of plasma samples, it will be necessary to repeat this analysis with more human samples in the future. Several peaks were observed in both plasma and urine samples before the retention time of the β-lapachone peak. Miao *et al.* [30] conducted β-lapachone metabolism study using human hepatocytes. Based on this study, we presume that these peaks are related to β-lapachone glucuronide-conjugated metabolites [30]. Further study is needed to identify the substance responsible for each peak before the β-lapachone peak.

In this study, LC-MS/MS analysis was conducted to evaluate the possibility of pH-dependent isomerization of β-lapachone in the human body. Isomerization of β-lapachone into α-lapachone was not observed in SGF after 1 hour, SIF after 3 hours, or in human plasma or urine samples, respectively. Through this study, the possibility of pH-dependent isomerization of β-lapachone in the human body was not confirmed.

Further studies are needed to analyze factors such as size reduction [25], complex formation [52-54], and water-soluble prodrug development [55] to improve the low solubility of β-lapachone, which limits its potential for drug development.

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