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Structural elucidation of the metabolites of lapachol in rats by liquid chromatography–tandem mass spectrometry

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ABSTRACT

Lapachol is a natural naphthoquinone compound derived from Bignoniaceae (*Tabebuia* sp.) that possesses a range of significant biological activities. Nine phase I and four phase II metabolites of lapachol in rat bile were firstly elucidated and identified using a sensitive LC-ESI–MS^n method. The molecular structures of the metabolites have been presented on the basis of the characteristics of their precursor and product ions, as well as their fragmentation mechanisms and chromatographic retention times. The results indicated that the phase I metabolites were predominantly biotransformed by the hydroxylation, semiquinone hydrogenation at the oxygen position or a side chain rearrangement. The phase II metabolites were identified as the glucuronidated conjugates which showed a characteristic neutral loss of 176 Da. Based on the results of this research, we have proposed the metabolic pathways for lapachol in rats. This work has provided novel information for the in vivo lapachol metabolism which could be used to develop a novel drug candidate, as well as a better understanding of the safety and efficacy of the drug.

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1. Introduction

Quinonoidal structures are widespread in nature and occur in almost all living organisms. Lapachol is a naturally occurring naphthoquinone that can be isolated from a number of different vegetal species, especially from those belonging to the Bignoniaceae family (*Tabebuia* sp.) [1,2]. Lapachol has been reported to exhibit a range of pharmacological activities, including antibiotic [3–5], antifungal [3,6], anti-inflammatory [7], antiprotozoal [8,9], antitumor [10,11], immunomodulatory [12], and interceptive [13] activities, which have been investigated and described for many years. To date, the main biological interest in lapachol has been focused on its activity and toxicity according to its role in mechanisms of oxidative stress induction, where it has been linked to the generation of reactive oxygen species, as well as the alkylation of cellular nucleophiles [14,15]. To reduce the unwanted toxicity of this material, as well as to improve its activity, several synthetic derivatives of lapachol have been designed and tested [16–18].

Although lapachol has been studied extensively and its many therapeutic applications have already been evaluated in clinical experiments, there has only been one article published in the literature pertaining to the in vitro metabolism of lapachol [19]. Furthermore, no work has been reported in the literature concerning the development of an LC–MS^n method for the in vivo evaluation of the metabolism of lapachol. The availability of a method capable of the determination and identification of *in vivo* metabolic profile of lapachol is very important for developing a better understanding of its pharmacological action and ensuring an efficient level of therapeutic application. The aim of the current work is to develop a sensitive and specific LC-ESI–MS^n method for the quantification and identification of the metabolites of lapachol in rats. In the current paper, nine phase I and four phase II metabolites of lapachol were found in rat bile, and the structures of these compounds were subsequently elucidated. Interestingly, the majority of these metabolites have never been reported before. The structures of these metabolites were characterized on the basis of their precursor and product ions, as well as their fragmentation mechanisms and HPLC retention times. The biotransformation pathways of lapachol in rats were elucidated on the basis of the results of an in vivo metabolic study. This investigation could also lead to the development of our overall understanding of the metabolic mechanisms and intermediate processes involved with these quinonoidal compounds.

2. Experimental

2.1. Chemicals and reagents

Lapachol [2-hydroxy-3-(3′-methyl-2′-butenyl-)-1,4-naphthoquinone] was purchased from Sigma Aldrich (St. Louis,

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MO, USA). The purity of the lapachol was greater than 98%. Methanol and acetonitrile were purchased from Fisher Scientific (Fair lawn, NJ, USA) as the HPLC grades. Acetic acid was purchased from Dikma Reagent Company (Beijing, China) as the HPLC grade. The water used in the current study was triply distilled. All of the other chemicals, reagents and solvents used were purchased as the analytical grades.

2.2. Apparatus and analytical conditions

The LC-ESI–MS system used in the current study consisted of a HPLC system (Series 1100, Agilent technology, Palo Alto, CA, USA) including a G1312A binary pump, a G1379A vacuum degasser and G1313A autosampler. The HPLC system was coupled to the Finnigan LCQ Deca XP ion-trap spectrometer equipped with electrospray source (Thermo Finnigan, San Jose, CA, USA). The LC-ESI–MS system was controlled using version 1.3 of the Xcalibur® software. The analytes were separated on a Capcell Pak MG C_{18} column (100 mm × 2 mm, i.d., 5 μm; Shiseido, Japan) coupled with a C_{18} guard column (10 mm × 2 mm, i.d., 5 μm; Shiseido) at ambient temperature.

The mobile phase was composed of methanol (A) and water (B), and was pumped at a flow-rate of 0.2 mL/min. The gradient programmer was performed in the following manner: 45% A at 0–5 min, 70% A at 25–35 min, 80% A at 35–40 min. A sample injection volume of 5 μL was used with a run time of 40 min for each sample. The eluent was in-line transferred to the ESI/MS system without any splitting. The mass spectrometric experiments were performed on an LCQ Deca-XP ion trap mass spectrometer (Thermo Finnigan), equipped with an electrospray ion source working in the negative ion mode. Nitrogen was used as a sheath gas and an aux/sweep gas, and ultra-high purity helium was used as collision gas for the collision induced dissociation (CID) experiments in the ion trap. Following a period of optimization, the ESI–MS was operated at the sheath flow rate of 30 psi with an ion spray voltage of 4 kV, and a heated capillary temperature of 350 °C, The MS^n product ion spectra were produced by collision induced dissociation of the deprotonated molecular ion $[M-H]^-$ of each analyte at their respective HPLC retention times with an isolation width (i.e., an m/z value) of 1. The collision energy for the analytes was in the range of 30–40%, depending on the structures of the different compounds.

2.3. Sample preparation

Adult male Sprague–Dawley rats were obtained from the Laboratory Animals Center at the Capital Medical University (Beijing, China). All of the animal studies were conducted in accordance with the guidelines provided by the Committee for the Care and Use of Laboratory Animals in China [20,21]. The rats (250–280 g, $n = 6$) were fasted for 12 h with free access to water prior to the administration of the lapachol. The rats were divided into two groups. The first group was orally administered with a suspension of lapachol in normal saline coupled with 1% Tween-80 at a dose of 34 mg/kg. The second group was treated as a control group and orally administered with the same volume of 1% Tween-80 without lapachol. And the sampling volume and preparation condition of the blank control bile are identical with that of the administration bile. The rats were subsequently anesthetized with urethane (20%) by intraperitoneal injection (0.5 mL/100 g), before being fixed on a wooden plate. An abdominal incision was made and the PE-10 tubing (0.20 inch i.d. × 0.37 inch o.d., Helix Medical, USA) was implanted in the rat bile duct to allow for the collection of bile samples. The bile samples were collected for 12 h, and all of the samples were stored at −80 °C prior to their analysis.

The bile samples of three rats was collected and combined, total volume is about 30 mL. After three-fold volume of ethyl acetate was added to the bile samples, the upper organic layers were collected and evaporated to dryness under a stream of nitrogen at 37 °C to give a residue. The lower aqueous layers were treated with methanol for protein precipitation, vortexed for 2 min, centrifuged at 7000 × g for 10 min, and collected the upper layers, after per-freezed for 24 h at −80 °C, the aqueous layers was freeze-dried by lyophilizer. The two phase residue samples containing the metabolites were re-dissolved in 200 μL of acetonitrile, filtered with 0.22 μm of membrane, and analyzed by LC-ESI–MS^n, respectively.

3. Results and discussion

3.1. Mass fragmental analysis of lapachol

To study the characterization of the mass spectra of the parent drug, a solution of 100 ng/mL of lapachol in acetonitrile was prepared and injected directly into the MS. Following a period of optimization using the automatic tune procedure, the maximum abundance value for the ions of lapachol was achieved. The LC–MS chromatogram and the MS/MS spectrum of lapachol are shown in Fig. 1. The mass spectral patterns of the parent drug served as templates in elucidation of the structures of the proposed metabolites of lapachol, in that the characteristic product ions and fragmentation rules of the parent drugs were used to identify the metabolites of the parent drug lapachol. Fragmentation of the deprotonated molecule ion of lapachol in the MS ion trap yielded four main product ions with m/z values of 226, 223, 213 and 186. The product ions with m/z values of 226 and 223 were formed through the loss of H_2O and CH_3 from the deprotonated molecular ion with an m/z value of 241, respectively. The product ion with an m/z value of 213 was formed through the loss of a CO fragment. The most abundant product ion provided an m/z value of 186 (2-hydroxy-3-methyl-1, 4-naphthoquinone anion). This m/z value was 40 Da less than the product ions formed with an m/z value of 226, and corresponded to the loss of a C_3H_4 fragment from this particular fragment [24]. The ion with an m/z value of 213 was further subjected to MS^3 analysis and produced two ions with m/z values of 198 and 158 through the loss of CH_3 and C_3H_4, respectively. The ion with an m/z value of 223 fragmented further to form ions with m/z values of 208, 205 and 195 through the loss of CH_3, CO and H_2O, respectively. The fragmentation pathways proposed for lapachol on the basis of these results have been presented in Fig. 2.

The mass spectrometric analysis of lapachol and its metabolites in the negative mode allowed for the characteristic deprotonated molecules to be obtained together with the fragmentation mechanisms of these anions. The occurrence of processes resulting in the elimination of radical species from the deprotonated molecule indicated that the even-electron rule was not being conserved, leading to the production of ions with m/z values of 226 and 186. These results were particularly important and allowed us to establish a platform for the analysis of the metabolites derived from lapachol. These results were therefore subsequently employed in the analysis of the drug metabolism of lapachol. The characteristic product ions and neutral losses from the parent compound can be used to identify the metabolites formed in vivo from lapachol.

3.2. Identification of the metabolites of lapachol

This part of the investigation focused on the characterization of the ESI–MS properties of the parent drug and its metabolites. The full scan mass spectra of the metabolite samples were compared with those of the blank and the parent drug to identify the proposed metabolites. These compounds were analyzed using LC-ESI–MS^n methods. Following the determination of the retention times and

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