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In vivo and in vitro metabolism of lefucoxib in rats

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ABSTRACT

The in vivo and in vitro biotransformation study of lefucoxib, 5-(3,4-dimethyl-phenyl)-1-methanesulfonyl-3-trifluoromethol-pyrazole, a new cyclooxygenase-2 (COX-2) inhibitor, was investigated in rats. To conduct the in vivo metabolism study, Wistar rats received lefucoxib in an oral dose, then their plasma and excreta were collected and analyzed. Through HPLC coupled with fluorescence detector and LC–MS^n analysis, hydroxylation was found to be the primary metabolism pathway of lefucoxib in rats. The chemical structure of the di-hydroxy metabolite was identified by MS^n spectra, and it was detected in rat plasma, urine and feces after an oral dose. However, the chemical structure of mono-hydroxy metabolites could not be identified by MS^n analysis due to the existence of two similar methyls on the phenyl ring of rofecoxib. To solve this problem, in vitro metabolism studies with liver microsome incubation helped accumulate enough metabolites for ^{1}H NMR analysis, which was employed and proved to be successful. Through further analysis of ^{1}H–^{1}H correlated spectroscopy (^{1}H–^{1}H COSY), chemical structures of two isomeric metabolites (mono-hydroxy metabolites) which had the same retention time in chromatograms were identified. The quantitive ratio of the two isomeric metabolites was also clarified to be 1:2 after analysis of the integrating height of ^{1}H NMR signals. Additionally, the present study illustrated the co-application of in vivo and in vitro metabolism on drug metabolite identification.

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1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used for the treatment of fever, pain, and inflammation. Their mechanism of action involves the inhibition of the enzyme cyclooxygenase (COX), which exists as two isoforms: a constitutive form, designated as COX-1, and an inducible form, referred to as COX-2 [1–3]. The constitutive COX-1 is widely expressed in nearly all tissues throughout the body [4,5], generating prostaglandins (PGs) that function to protect the gastric mucosal lining, regulate blood flow to the kidney, and support platelet aggregation. In contrast, COX-2 is constitutively expressed in limited healthy tissues, e.g., kidney, and is predominantly expressed in inflamed tissues [6]. However, when induced, the PGs produced by COX-2 are associated with the pain and swelling of inflammation. Now, it is well known that the therapeutic utility of nonselective COX inhibitors is due to inhibition of COX-2, while their side effect profile (i.e., gastrointestinal irritation and bleeding) results from inhibition of COX-1. Clearly, the development of selective inhibitors of COX-2 is an attractive target, because such agents retain the anti-inflammatory, analgesic, and antipyretic properties of nonselective COX inhibitors, while reducing the risk of gastrointestinal side effects [7–9]. Until recently, much attention has been focused on the design of potent and selective COX-2 inhibitors, such as celecoxib, etoricoxib and valdecoxib.

Lefucoxib (5-(3,4-dimethyl-phenyl)-1-methanesulfonyl-3-trifluoromethol-pyrazole) (Fig. 1) is a new selective inhibitor of COX-2, and it is developed to treat the signs of osteoarthritis and the symptoms of rheumatoid arthritis. The incidence of gastrointestinal complications caused by lefucoxib is significantly lower than that of the nonselective NSAIDs (lefucoxib selective inhibition ratio of COX-2 over COX-1 was observed to be approximately 450, and the efficacy study of lefucoxib will be detailed elsewhere later). In previous studies conducted in our laboratory, the pharmacokinetics and excretion of lefucoxib were examined in rats [10]. However until recently, no metabolism studies on lefucoxib had been reported. To clarify the metabolism profile of lefucoxib in rats, the present studies were designed and performed.

2. Materials and methods

2.1. Chemicals and dosing solutions

Lefucoxib was provided by China PLA General Hospital (Beijing, China). $NADP^+$ and Tris were purchased from Sigma-Aldrich (St.

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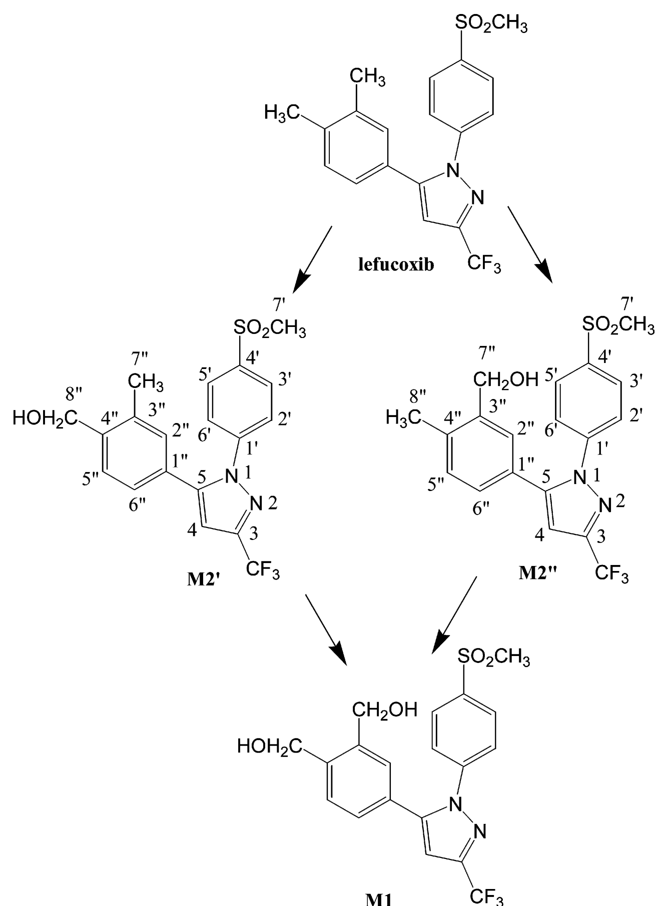

Fig. 1. Metabolism pathways of lefucoxib.

Louis, MO). HPLC grade methanol and acetonitrile were obtained from Fisher Scientific (Pittsburgh, USA). HPLC grade water was produced by the Millipore Direct-Q system. Methyl *tert*-butyl ether (HPLC grade) was obtained from Mallinckrodt Baker, Inc. (Phillipsburg, USA). Magnesium chloride and sodium hydroxide (analytical grade) were bought from Beijing Beihua Fine Chemicals Company, Limited (Beijing, China). All other commercially available reagents and solvents were of either analytical or HPLC grade.

2.2. Animal studies

7-week-old male Wistar rats weighing approximately 220–250 g were obtained from the Laboratory Animal Center in the Academy of Military Medical Science (Beijing, China). Prior to the experiment, all rats were housed in temperature-controlled rooms (23–25 °C) with a 12-h light/dark cycle. After 2 weeks of adaptation to the environment, the rats were used for the metabolic studies and preparation of liver microsomes.

2.3. Metabolic studies in vivo

The in vivo metabolic study of lefucoxib was carried out in five rats. Before administration, the rats had fasted for 12 h but were allowed access to water throughout the experiment. Animals were given food 3 h after lefucoxib oral administration. Lefucoxib was suspended in a 0.5% carboxymethyl-cellulose solution, and then administered via gastric gavage at a dose of 75 mg kg^{-1}. Blood samples (approximately 0.4 ml) were collected in heparinized polythene tubes before administration and post-dose at 3.0, 6.0, 12.0, and 24.0 h. Plasma was separated out by centrifugation at 6000 rpm for 10 min. Feces and urine were collected before and after administration in the following time intervals: 0–12 h, 12–24 h, 24–36 h and 36–48 h. Sample aliquots collected during the same time intervals were pooled and then stored at −20 °C until analysis.

2.4. Metabolic studies in vitro

2.4.1. Preparation of microsomes from rat liver

The preparation of liver microsomes was conducted using the method of Elovaara et al. [11] with some modifications. Briefly, livers (n = 5) were perfused in situ with ice-cold normal saline, then removed, weighed and homogenized in 4 volumes (w/v) of ice-cold 50 mM Tris–HCl (pH 7.4) buffer containing 0.250 M sucrose. Enzyme fractions were prepared at 4 °C by differential ultracentrifugation. The supernatant of the first centrifugation (10,000 × g, 15 min) was run at 100,000 × g for 60 min to obtain the microsome fraction pellets. The pellets were resuspended in the Tris–HCl/sucrose buffer (mentioned above), and then 10% (v/v) glycerol was added. Next, the resuspended liver microsome solution was divided into small aliquots, and stored in Eppendorf tubes at −80 °C until use. Protein concentrations of liver microsomes were determined using the Pierce bicinchoninic acid (BCA) protein reagent kit (Pierce Chemical, Rockford, IL).

2.4.2. Liver microsome incubation studies

To conduct in vitro metabolism studies and accumulate enough metabolites used for ^{1}H NMR analysis, microsome incubation was performed. The incubation was conducted at 37 °C in a shaking water bath (50 cycle min^{-1}), using a 500 ml beaker. Briefly, the final assay volume was 150 ml and consisted of the following: 50 mM Tris–HCl buffer (pH 7.4), magnesium chloride (5 mM), microsome protein (0.2 mg ml^{-1}), NADPH generating system and lefucoxib (1 mM) which was added in the form of acetonitrile solution (acetonitrile accounts for 1% (v/v) in the final incubation mixture). The NADPH generating system consisted of 10 mM glucose 6-phosphate, 0.5 mM NADP$^+$, and 1 unit of yeast glucose 6-phosphate dehydrogenase ml^{-1} (final concentrations). The reaction was initiated by addition of the NADPH generating system after a 3-min preincubation at 37 °C, and then terminated by treating the beaker with an ice-cold water bath after a 1-h incubation period. Incubation mixture was stored at −20 °C until analysis.

2.5. Biological samples preparation

50.0 μl sodium hydroxide solution (1 mol l^{-1}) and 500.0 μl methyl *tert*-butyl ether were added to 200.0 μl of biological matrices (plasma, urine and fecal homogenate, fecal samples were homogenized with water in the ratio of 1:3 (w/w) to obtain fecal homogenate), respectively. The mixture was vortex mixed for 1 min and centrifuged at 10,000 × g for 5 min, then the supernatant layer was transferred to new 1.5 ml Eppendorf tubes. The subnatant solution was extracted with 500.0 μl methyl *tert*-butyl ether again, and the supernatant was combined with the former. The combined organic layer was dried under a stream of nitrogen gas at 40 °C. The residue was reconstituted in 80.0 μl of a mixed solution (methanol–water, 80:20, v/v) and 50.0 μl was applied to the HPLC apparatus. Liver microsome incubation samples were extracted with the same method, except the treating volume was increased by 40 times in 50 ml centrifuge tubes.

2.6. HPLC

HPLC analysis was carried out using Agilent 1100 HPLC system consisting of G1322A Vacuum Degasser, G1311A Quat

Pump, G1316A Thermostatted Column Compartment, G1313A Autosampler, G1321A Fluorescence Detector and G2170AA single instrument ChemStation for liquid chromatography systems. Chromatographic separation was achieved on a Kromasil C_{18} column (250.0 mm × 4.6 mm, Eka Chemicals, Sweden). Elution was carried out using a gradient as follows: 0–6 min, 60% methanol, 40% water; 6–8 min, 60% methanol, 40% water → 80% methanol, 20% water; 8–16 min, 80% methanol, 20% water; 16–17 min, 80% methanol, 20% water → 100% methanol; 17–25 min, 100% methanol; 25–27 min, 60% methanol, 40% water, and the flow rate was set at 1 ml min^{-1}. The temperature of the thermostated oven containing the column was set at 20 °C and the injection volume was 50.0 μl. Fluorescence detection was performed at an excitation wavelength of 254 nm and an emission wavelength of 430 nm. After chromatographic separation, metabolites of lefucoxib eluted by the mobile phase at different retention times were collected, respectively, then dried under a stream of nitrogen gas at 40 °C and used for LC–MS^n and ^{1}H NMR analysis.

2.7. LC–MS^n analysis

Qualitative analysis of lefucoxib and its metabolites was carried out by MS^n spectra with a ThermoFinnigan LCQ Advantage Quadruple Ion Trap Mass Spectrometer. M0 (lefucoxib), M1 (dihydroxy metabolites) and M2 (mono-hydroxy metabolites) were dissolved in a solution containing 80% methanol and 20% water, and then introduced to mass spectrometer by a syringe pump to get fragmentation pathways. Spectrometry experiments were performed with ESI in positive ion mode. The capillary voltage was fixed at 40 V, and its temperature was maintained at 280 °C. The spray voltage was set at 2.0 kV. N_2 was used as both the sheath and auxiliary gas at flow rates of 28 and 15 units, respectively (units specific to ThermoFinnigan systems). The MS^n spectra were produced by collision-induced dissociation (CID) of the selected precursor ions with He, and the relative collision energy was set at 40% (units specific to ThermoFinnigan systems).

2.8. NMR analysis

Samples (M2) purified from the liver microsome incubation mixture were analyzed using deuterated methanol (CD_3OD) solution in a 5-mm concentric capillary tube and an INOVA 600 (600 MHz, Varian, Palo Alto, CA, USA) instrument with a 5-mm indirect detection probe. Chemical shifts were reported in parts per million (δ) downfield from tetramethylsilane.

3. Results

3.1. In vivo and in vitro metabolite profiles by HPLC analysis

Plasma, urine and feces collected after oral administration and liver microsome incubation mixtures were analyzed by HPLC. Compared with blank samples, the compounds detected by fluorescence detection included the parent drug and two products believed to be drug-related, with retention times of 8.3 (M1) and 11.9 min (M2) in chromatograms. In plasma and feces, M1, M2 and unchanged lefucoxib all presented. In urine, M1 and M2 were detected, whereas in the liver microsome incubation mixtures, the analysis revealed the presence of M2 and lefucoxib (representative chromatograms are shown in Fig. 2).

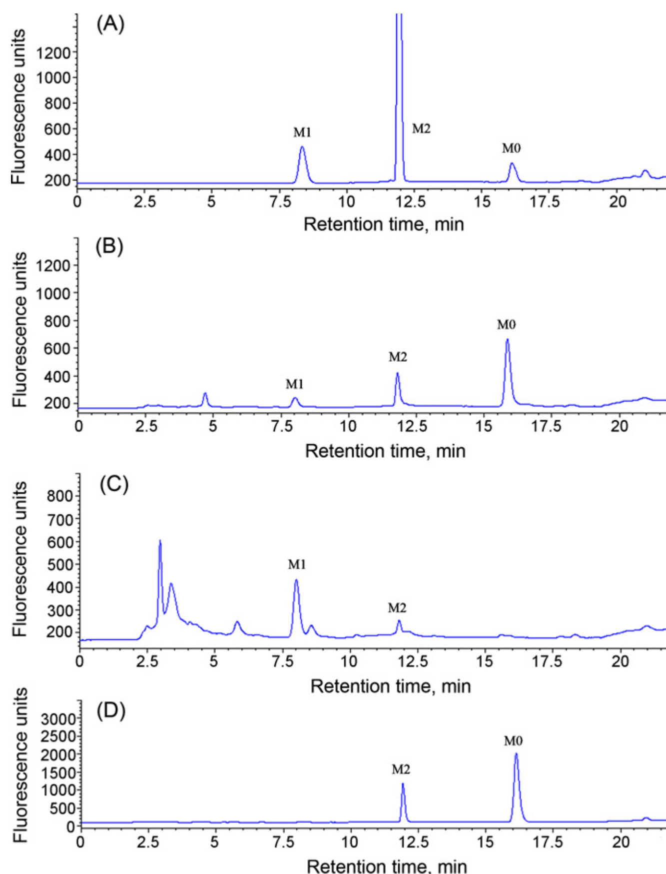

Fig. 2. (A) Representative chromatograms of rat plasma after administration of lefucoxib. (B) Representative chromatograms of rat feces after administration of lefucoxib. (C) Representative chromatograms of rat urine after administration of lefucoxib. (D) Representative chromatograms of rat liver microsome incubation samples.

3.2. LC–MS^n analysis of lefucoxib metabolites

3.2.1. Parent drug (M0)

M0 was found in plasma, feces and microsome incubation samples. Mass spectra of M0 showed a $[M+H]^+$ ion at m/z 395, and its MS^n spectra showed a characterized fragmentation pathway of m/z: 395 → 375 → 296 (loss of HF and $-SO_2CH_3$, respectively), which was identical with the authentic standard of lefucoxib. All these data indicated that M0 was lefucoxib, which was confirmed by the comparison of retention time of M0 and lefucoxib standard.

3.2.2. Metabolite M1

M1 presented in feces, urine, and plasma. Mass spectra of M1 (Fig. 3(a)) showed a $[M+H]^+$ ion at m/z 427, which was 32 Da higher than that of lefucoxib, suggesting the presence of two hydroxyl groups. Its MS^n spectra (Fig. 3(b) and (c)) showed two characterized fragmentation pathways which were analyzed as follows. One fragmentation pathway of m/z 427 → 407 → 328 (loss of HF and $-SO_2CH_3$, respectively) was identical with that of lefucoxib, confirming that it was a drug-related product, and the other pathway of m/z 427 → 409 → 391 (loss of 18 Da, the molecular weight of H_2O every fragmentation) indicated the presence of two hydroxy groups. Taken together, M1 was identified as the dihydroxy metabolite of lefucoxib, and the chemical structure of M1 was shown in Fig. 1.

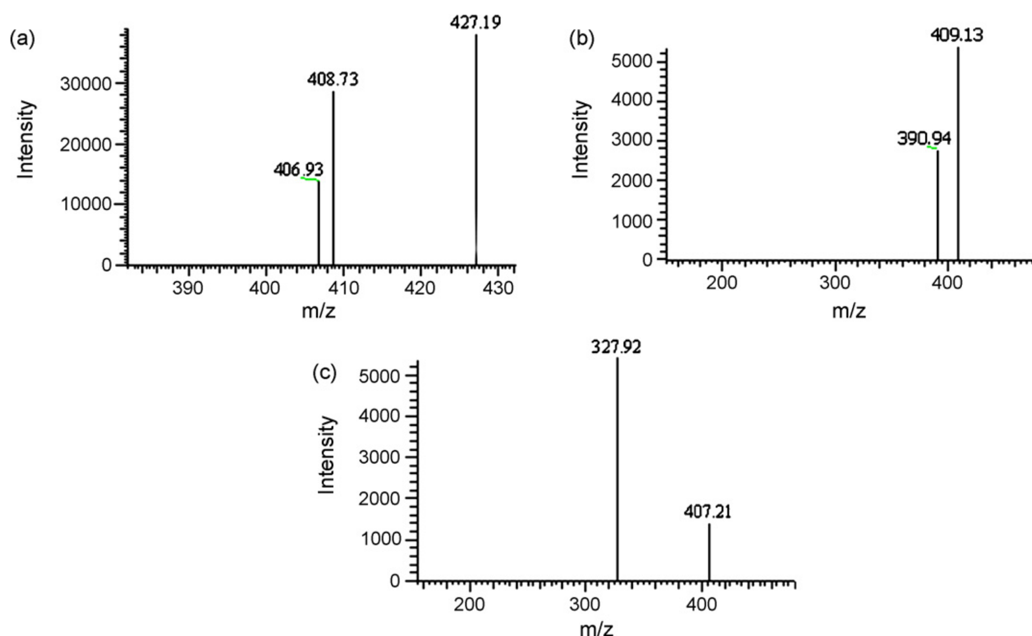

Fig. 3. Fragmentation pathway of M1.

3.2.3. Metabolite M2

M2 presented in feces, urine, plasma and the microsome incubation mixture. Mass spectra of M2 (Fig. 4(a)) showed a $[M+H]^+$ ion at *m/z* 411, which was 16 Da higher than that of lefucoxib, indicating the presence of one hydroxyl group. Its MS^n spectra (Fig. 4(b)) showed a characterized fragmentation pathway, which was *m/z* 411 → 391 → 312 (loss of HF and $-SO_2CH_3$, respectively). This pathway was identical with that of lefucoxib, confirming that it was a metabolite of lefucoxib. Another fragmentation pathway of *m/z* 411 → 393 (loss of 18 Da, the molecular weight of H_2O, Fig. 4(a)) confirms the presence of a hydroxyl group. However, the position of the hydroxyl group could not be deduced due to the presence of two similar methyl groups on the phenyl ring of lefucoxib. The probable chemical structures were shown in Fig. 1 (assigned as M2′ and M2″).

3.3. ^{1}H NMR analysis of Lefucoxib metabolites

The position of the hydroxyl group on M2 was identified by ^1H–^1H correlated spectroscopy (^1H–^1H COSY), which was shown in Fig. 5(a). It was indicated that the proton signal at δ 7.15 (C–2″H, M2′) correlated with the proton signal at δ 2.28 (C-7″H, M2′); moreover, the proton signal at δ 7.38 (C-5″H, M2′) correlated with the proton signal at δ 4.63 (C-8″H, M2′). Thus, the position of the hydroxyl group could be elucidated at C-8″ (Fig. 1), which proved the existence of M2′. While the proton signal at δ 7.36 (C-2″H, M2″) correlated with the proton signal at δ 4.58 (C-7″H, M2″); the proton signal at δ 7.17 (C-5″H, M2″) correlated with the proton signal at δ 2.32 (C-8″H, M2″). Therefore, the position of the hydroxyl group could be elucidated at C-7″ (Fig. 1), which proved the existence of M2″. Based on the above analyses, it could be concluded that M2 was not a pure compound, but a mixture containing two isomeric mono-hydroxy metabolites of lefucoxib, i.e. M2′ and M2″.

In addition, the ^{1}H NMR spectra and data analyses of the two mono-hydroxy metabolites were presented in Fig. 5(b) and Table 1, respectively. Together with the integrating height of ^{1}H NMR signals, it could be deduced that the quantitive ratio of M2′ to M2″ was 1:2.

3.4. Metabolite pathway

All above studies indicated that lefucoxib underwent oxidation metabolism, i.e. hydroxylation on the methyl group in rats, both in vivo and in vitro. After hydroxylation, the polarity of lefucoxib metabolites became stronger than that of the parent drug, and the increase in polarity allowed the metabolites to be more easily excreted from the body. This conclusion was further supported by the relatively short retention time of M1 and M2 in the chromatograms. In addition, a preliminary conclusion could be drawn by analyzing the height and area of chromatography peaks in

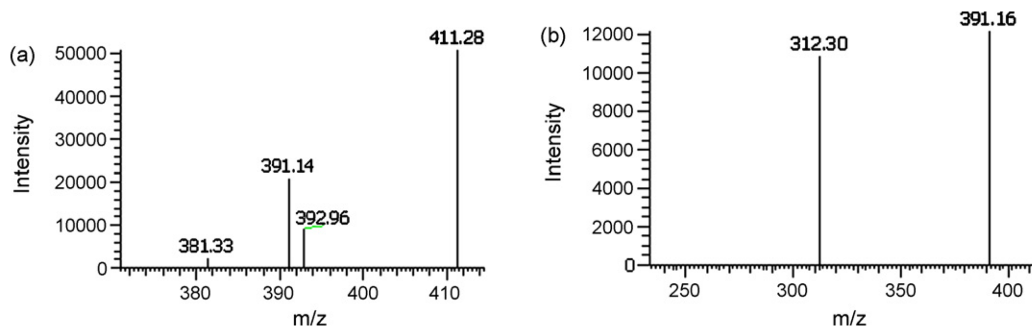

Fig. 4. Fragmentation pathway of M2.

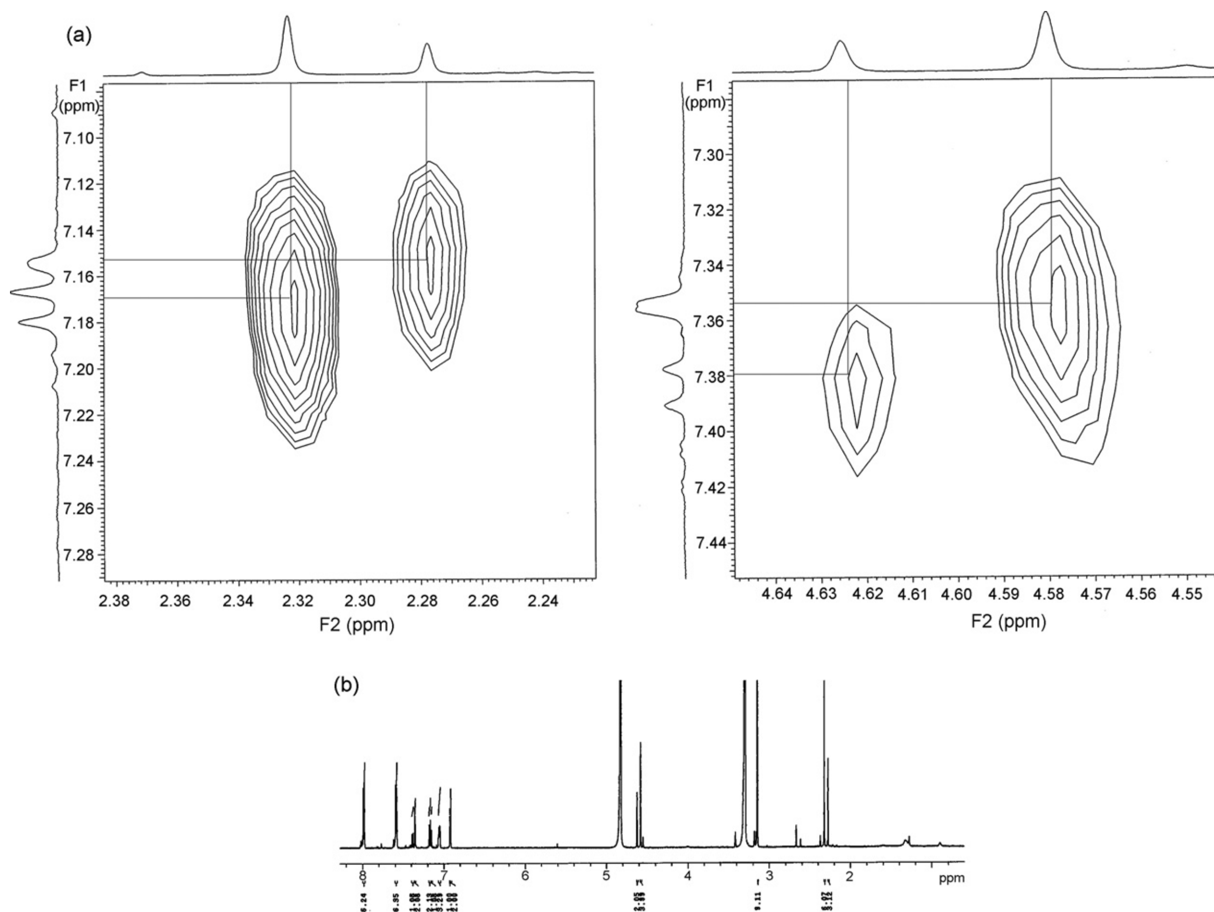

Fig. 5. ^{1}H–^{1}H COSY and ^{1}H NMR spectra of mono-hydroxy metabolites (M2).

chromatograms. It seemed that the mono-hydroxylefucoxib (M2) predominated in plasma and feces, while the di-hydroxylefucoxib (M1) accounted for the majority in urine.

4. Discussion

The results of the present study indicated that hydroxylation was the primary metabolism pathway of lefucoxib in rats, both in vivo and in vitro. However, unlike other selective inhibitors of COX-2 such as rofecoxib whose hydroxyl metabolites and the corresponding glucuronide conjugate metabolites were all detected with HPLC coupled with fluorescence detection in rat urine [12], only hydroxyl metabolites of lefucoxib were detected in rat urine, feces, plasma and liver microsome incubation samples. Initially, the failure of detecting lefucoxib glucuronide conjugate metabolites was thought to be caused by inappropriate biological sample preparation methods. Therefore, many preparation methods were tried, such as protein precipitation with acetonitrile [12], in which glucuronide conjugate metabolites of rofecoxib were determined. However, only hydroxyl metabolites of lefucoxib were detected. Besides, solid-phase extraction was also applied to determine the secondary metabolites. Still, no glucuronide conjugate metabolites of lefucoxib were found.

Table 1
^{1}H NMR data of mono-hydroxy metabolites (M2)

Protons	Proton chemical shift (δH)	
	M2′	M2″
3′, 5′	7.97–8.00 (2H, d)	7.97–8.00 (4H, d)
2′, 6′	7.58–7.60 (2H, d)	7.58–7.60 (4H, d)
5″	7.38 (1H, d)	7.17 (2H, d)
2″	7.15 (1H, d)	7.36 (2H, s)
6″	7.04–7.07 (1H, d)	7.04–7.07 (2H, d)
4	6.93 (1H, s)	6.92 (2H, s)
8″	4.63 (2H, s)	2.32 (6H, s)
7″	2.28 (3H, s)	4.58 (4H, s)
7′	3.15 (3H, s)	3.15 (6H, s)

It was reported that the metabolic pathway for celecoxib, another selective inhibitor of COX-2, involved oxidation of the aromatic methyl group to form a hydroxy metabolite, which underwent additional oxidation to a carboxylic acid metabolite in rats [13]. Another study reported that carboxylic acid metabolite of celecoxib could be determined by HPLC–UV [14], which was tried and thought to be helpful in detecting carboxylic acid metabolites of lefucoxib if they existed. But carboxylic acid metabolites were still not found in rat urine and feces.

To profile the complete metabolism pathway, LC–MS was used instead of HPLC. Still, only hydroxyl metabolites were detected.

It seems that, lefucoxib might not undergo carboxylic acid metabolism in rats due to the formation of its very polar metabolites, di-hydroxy metabolites, which differed greatly from the mono-hydroxy metabolites of other COX-2 inhibitors. This difference in metabolism pathway might be caused by the different number of methyls in their chemical structures. There were two methyls on the phenyl ring of lefucoxib, however, only one methyl presented on the phenyl ring of celecoxib and etoricoxib, and no methyl appeared on the phenyl ring of rofecoxib. To confirm this conclusion, further metabolism study using radiolabeled lefucoxib is needed.

In addition, the present study illustrated the co-application of in vivo and in vitro metabolism in identification of drug metabolites. It was well-known that metabolites had better therapeutic effects and lower toxicity than parent drugs in some cases. Therefore, it was necessary to clarify their chemical structures so as to make their further efficacy and toxicological study possible in the process of drug research and development. There is no doubt that LC–MS may provide valuable information in chemical structure identification. However, sometimes analysis with NMR is indispensable, such as in the present case. Generally, NMR analysis required milligram levels of analyte which could not be obtained from body fluid. In this situation, in vitro metabolism methods may be able to accumulate many more metabolites than in vivo methods, offering a reasonable and practicable choice.

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