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Isolation of an unknown metabolite of the non-steroidal anti-inflammatory drug etodolac and its identification as 5-hydroxy etodolac

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Abstract

The non-steroidal anti-inflammatory drug etodolac is extensively metabolized in the liver. Renal elimination of etodolac mainly as glucuronide and its other phase I and phase II metabolites is the primary route of excretion. High-performance liquid chromatography assays of human urine after application of etodolac indicated the existence of a further monohydroxylated metabolite (metabolite X) that was identified as 5-hydroxy etodolac. For the identification, electrospray ionization mass spectrometry (ESI-MS) as well as ^{1}H-nuclear magnetic resonance (^{1}H-NMR) and ^{13}C-NMR spectroscopy have been used. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: 5-Hydroxy etodolac; Etodolac; Phase I metabolites; High-performance liquid chromatography; Electrospray ionization mass spectrometry; Nuclear magnetic resonance spectroscopy

1. Introduction

Etodolac (ETO) (1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-*b*]-indole-1-acetic acid) (Fig. 1), a non-steroidal anti-inflammatory drug, is primarily used in the therapy of rheumatic diseases and postoperative pain [1]. It is rapidly metabolized in the liver, followed by renal elimination as the primary route of excretion. Until now, the monohydroxylated metabolites 6-hydroxy-, 7-hydroxy- and 8-(1′-hydroxyethyl) etodolac could be identified (Fig. 1). Furthermore, the acyl glucuronides of this monohydroxylated metabolites as well as etodolac glucuronide were found [2,3]. In addition, Ferdinandi et al. reported on the existence of 4-ureido etodolac [4]. In our investigation, we were able to isolate the postulated monohydroxylated metabolite (metabolite X) [5] from human urine with a semi-preparative RP-18 column and to identify it as 5-hydroxy etodolac (Fig. 1) by electrospray ionization mass spectrometry (ESI-MS), ^{1}H-nuclear magnetic resonance (NMR) and ^{13}C-NMR spectroscopy.

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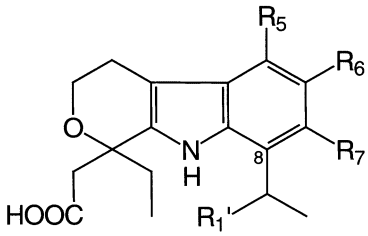

	R_5	R_6	R_7	$R_{1'}$
etodolac	H	H	H	H
5-hydroxy etodolac	OH	H	H	H
6-hydroxy etodolac	H	OH	H	H
7-hydroxy etodolac	H	H	OH	H
8-(1'-hydroxyethyl) etodolac	H	H	H	OH

Fig. 1. Structure of etodolac (ETO), its postulated metabolite 5-hydroxy etodolac (5-OH-ETO) and its known metabolites 6-hydroxy etodolac (6-OH-ETO), 7-hydroxy etodolac (7-OH-ETO) and 8-(1′-hydroxyethyl) etodolac (8-OH-ETO).

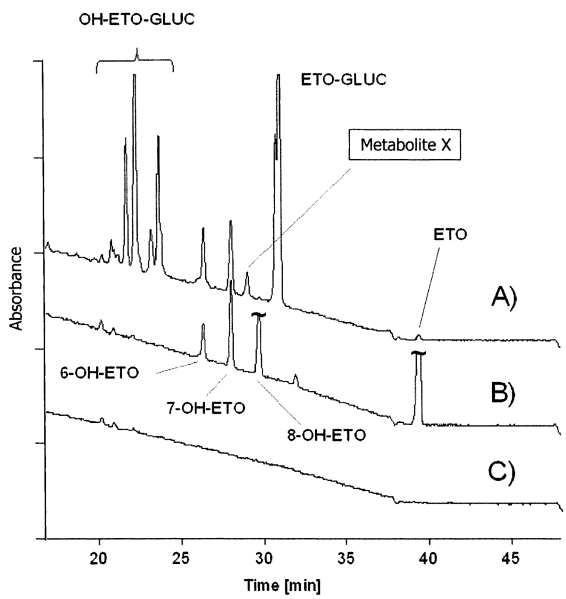

Fig. 2. HPLC chromatograms (gradient mode) of a (A) human urine sample after administration of 400 mg racemic etodolac, (B) blank urine sample spiked with 300 μg/ml ETO, 6-OH-ETO, 7-OH-ETO and 8-OH-ETO, and (C) blank urine sample. Column, LiChrospher® 100 RP-18 (5 μm), 4 × 250 mm; eluent, acetonitrile (A)/acetic acid (0.5%) (B); gradient: 0.0 min, 2% A; 35.0 min, 50% A; 45.0 min, 50% A; flow, 1.0 ml/min; injection, 10 μl; detection, UV 222 nm.

2. Experimental

2.1. Chemicals and reagents

Etodolac (Lodine® 100) tablets were purchased from Laboratoires Wyeth France (Paris, France). Racemic etodolac (ET) and its phase I metabolites, 6-hydroxy etodolac (6-OH-ET), 7-hydroxy etodolac (7-OH-ET) and 8-(1′-hydroxyethyl) etodolac (8-OH-ET) were gifts from Wyeth Pharma GmbH (Münster, Germany) and Wyeth Ayerst Research (Princeton, NJ, USA). Acetic acid, hydrochloric acid and acetonitrile were from different commercial sources and used without further purification. LiChrospher® 100 RP-18 (5 μm) used for the preparation of an analytical and a semi-preparative high-performance liquid chromatography (HPLC) column was a gift from Merck (Darmstadt, Germany), methanol-d_4 (Uvasol®) was purchased from the same source.

2.2. Analytical separation

The separation of etodolac and its phase I and phase II metabolites was performed with a LiChrospher® 100 RP-18 column (particle size, 5 μm; dimension, 4 × 250 mm) connected with a LiChrospher® 100 RP-18 guard column. The chromatographic system consisted of a L-6200 A Intelligent Pump (Merck Hitachi, Tokyo, Japan), a 655 A Variable Wavelength UV Monitor (222 nm) (Merck Hitachi, Tokyo, Japan) and a 655-61 Processor A (Merck Hitachi, Tokyo, Japan). The eluent was acetonitrile (A)/acetic acid (0.5%) (B) in a gradient mode (0.0 min, 2% A; 35.0 min, 50% A, 45.0 min, 50% A) at a flow rate of 1.0 ml/min. The injection volume was 10 μl.

For peak purity control, the same column system was used in an isocratic mode. The chromatographic system consisting of a Waters 2690 Separations Module (Milford, MA, USA) was coupled with the LCQ® ion trap mass spectrometer (Finnigan MAT, San Jose, CA, USA). The eluent was acetonitrile/acetic acid (0.5%) (34/66) at a flow rate of 1.0 ml/min. The injection volume was again 10 μl.

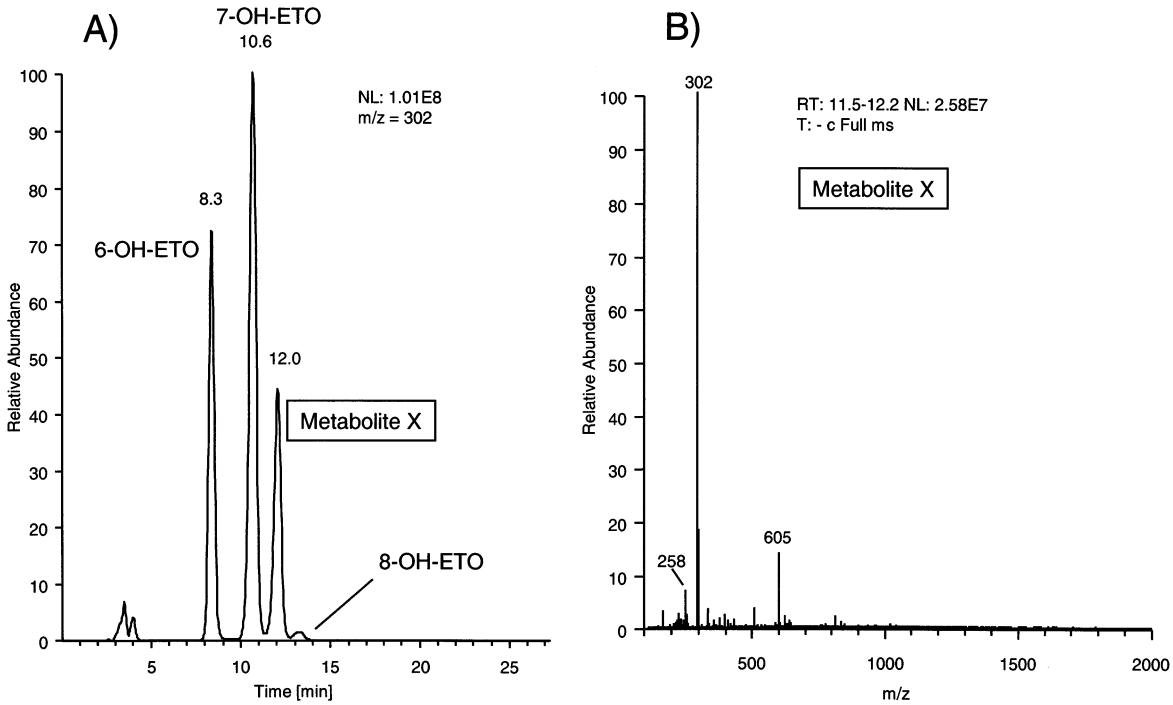

Fig. 3. HPLC–ESI-MS chromatogram (selected mass track, $m/z = 302$) (isocratic mode) (A) of a human urine sample (1–5 h) after administration of 600 mg racemic etodolac, and ESI-MS spectrum (B) of metabolite X. Column, LiChrospher® 100 RP-18 (5 μm), 4 × 250 mm; eluent, acetonitrile/acetic acid (0.5%) 34/66; flow, 1.0 ml/min; injection, 10 μl; detection, ESI-MS. ESI-MS conditions: polarity, negative; source voltage, 3.5 kV; sheath gas flow, 80 arbitrary units; heated capillary, 200°C; mass range, $m/z = 100–2000$.

2.3. Isolation of metabolite X

Urine was collected 1–5 h after oral administration of six tablets of 100 mg racemic etodolac (Lodine®). The sample (120 ml) was acidified with 1 N HCl to about pH 2–3, centrifuged with a P3-centrifuge (Phywe, Göttingen, Germany) and stored at 4°C under light protection.

Metabolite X was isolated directly from the urine sample on a LiChrospher® 100 RP-18 column (particle size, 5 μm; 7 × 250 mm) connected with a LiChrospher® 100 RP-18 guard column. The aforementioned HPLC set-up from Merck Hitachi was used. The eluent was acetonitrile/acetic acid (0.5%) in an isocratic mode (34/66) at a flow rate of 2.0 ml/min. The urine sample was injected in 3 ml portions. The fractions at a retention time of 23.2–23.8 min were collected, and the eluent was evaporated under vacuum, ambient temperature and light protection. After each run, the column was rinsed by an injection of 1000 μl acetonitrile. The raw sample of the metabolite was further purified in four runs by the same method. From these combined metabolite X fractions, acetonitrile was removed under vacuum. Subsequently, the solution was diluted by the addition of about 5 ml of water and lyophilizated with a LYOVAC GT 2 freeze-dryer (Finn-Aqua Santasaco-Sohlberg Corp., Tuusula, Finland).

2.4. Characterization of metabolite X by nano-ESI-MS

For the MS investigation, a laboratory-made nano-spray interface was used. About 5 μl collected fractions of metabolite X were diluted with the same volume of acetonitrile and introduced into the nano-spray capillary. After loading the capillary, a thin stainless steel wire was inserted

into the tip of the capillary to obtain the electrical contact of the sample solution with the electrospray voltage. The capillary was positioned 1–2 mm in front of the orifice of the MS inlet. The position of the nano-spray capillary could be manipulated in the x, y and z directions via micrometer screws. To launch the spray process, the capillary was slightly touched against the MS inlet and then centred in front of the orifice of the mass spectrometer. A potential of 1000 V was applied to the nano-spray capillary. A full scan spectrum (mass range, 100–2000) in negative ion mode was recorded.

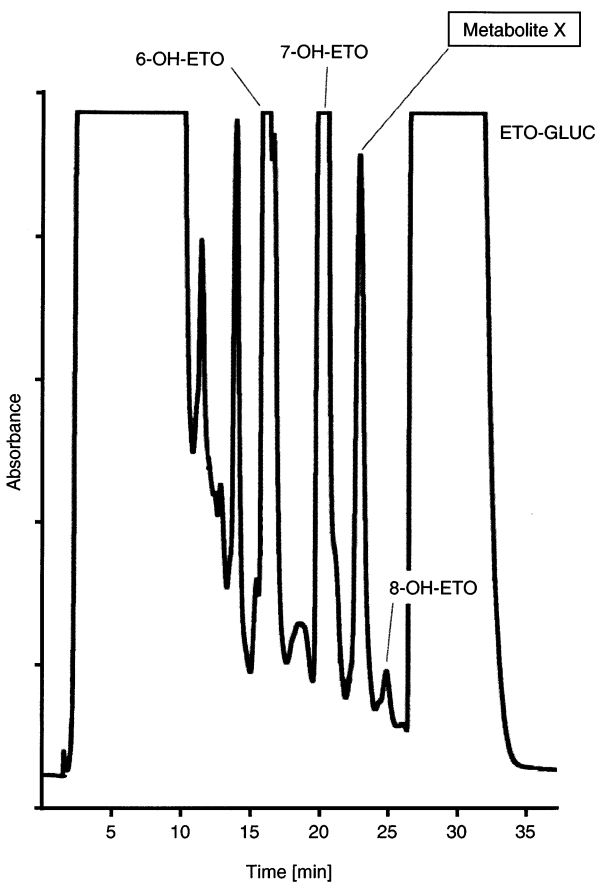

Fig. 4. HPLC chromatograms (isocratic mode) of a human urine sample (1–5 h) after administration of 600 mg racemic etodolac. Column, LiChrospher® 100 RP-18 (5 μm), 7 × 250 mm; eluent, acetonitrile/acetic acid (0.5%) 34/66; flow, 2.0 ml/min; injection, 3000 μl; detection, UV 222 nm.

2.5. Characterization of metabolite X by NMR spectroscopy

For the NMR investigations, a Gemini 200 NMR spectrometer (Varian, Palo Alto, CA, USA) was used. The ^{1}H-NMR spectra were recorded with 200 MHz and the ^{13}C-NMR spectrum with 50 MHz. The whole quantity of the isolated metabolite (about 100 μg) was dissolved in methanol-d_4. The methanol signal at $\delta = 3.3$ ppm ($C\underline{H}D_2OD$) served as internal standard. With this sample, a ^{1}H-NMR spectrum, a ^{1}H-COSY 45 long-range spectrum and a ^{13}C-NMR spectrum were acquired. For the ^{1}H-COSY long-range experiment, a pulse sequence from the literature [6–8] was modified. The second 90° pulse was replaced by a 45° pulse and the time $D_2(\Delta)$ was set to 0.2 s.

3. Results and discussion

3.1. Analytical separation

With the analytical gradient method, it was possible to separate etodolac and all hydroxylated metabolites. ETO, 6-OH-ETO, 7-OH-ETO and 8-OH-ETO (Fig. 1) could be identified by spiking a blank urine sample with the reference substances (Fig. 2). Chromatogram (A) shows that just a minor amount of 8-(1′-hydroxyethyl) etodolac is eliminated as the free phase I metabolite. Besides, the not completely separated diastereomers of etodolac glucuronide (ETO-GLUC) and the diastereomeric glucuronides of the hydroxylated metabolites (OH-ETO-GLUC) could be detected by this method. According to MS experiments, the additional peak at $t = 29.1$ min was postulated to be another monohydroxylated metabolite (metabolite X) [5]. For the isolation, an isocratic HPLC method was used. In order to check the peak purity before the isolation, the analytical column was connected with an electrospray ionization mass spectrometer. The chromatogram resulting from the selected mass track $m/z = 302$ (M^{-1}) (Fig. 3A) shows the three known hydroxylated metabolites and metabolite X, which was identified in this study as 5-hydroxy

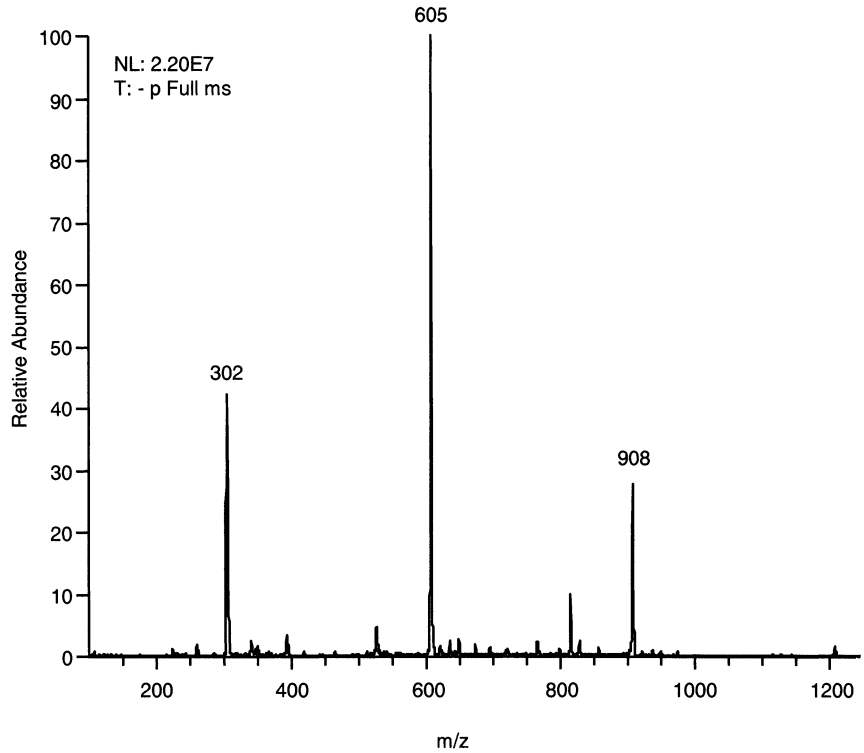

Fig. 5. Nano-ESI-MS spectrum of the purified metabolite X dissolved in acetonitrile/acetic acid (0.5%) 1:1. Interface, laboratory-made nano-spray interface. Nano-ESI-MS conditions: polarity, negative; source voltage, 1000 V; heated capillary, 40°C; displayed mass range, $m/z = 100–1200$.

etodolac. The mass spectrum (Fig. 3B) demonstrates that the peak purity of metabolite X is sufficient. The mass signal $m/z = 302$ corresponds to the anion of the metabolite. Typical for ESI-MS with anions, the dimer ($m/z = 605$) can also be observed. The signal at $m/z = 258$ results from a MS-induced fragment of the metabolite.

3.2. Isolation of metabolite X

For the isolation, the analytical isocratic method was applied on a semi-preparative RP-18 column connected with a UV detector (222 nm). The urine samples were acidified and centrifuged. No other sample pre-treatment was performed, because some of the metabolites are quite unstable especially under the influence of UV light. Thus, light protection was necessary. For each run, a volume of 3000 μl was injected. Because of the absence of organic modifiers in the urine sample, all metabolites were retained on the top of the column. This on-column pre-concentration resulted in well-separated peaks that could be fractionated quite easily (Fig. 4). An HPLC–ESI-MS analysis showed that even this raw fraction was quite pure. Nevertheless, the metabolite was purified again by the same method. After removal of the eluent, about 100 μg colourless substance were obtained.

3.3. Characterization of metabolite X by nano-ESI-MS

A small amount of the collected fractions after the purification was used for recording a mass spectrum using a laboratory-made nano-spray interface. The spectrum is depicted in Fig. 5, and shows the signal for the metabolite anion ($m/z = 302$), the dimer ($m/z = 605$) and even the trimer ($m/z = 908$).

3.4. Characterization of metabolite X by NMR spectroscopy

The proof for the structure of the postulated 5-hydroxy etodolac was performed by NMR experiments. The ^{1}H-NMR spectrum is presented in Fig. 6. The two doublets at $\delta = 6.64$ and 6.27 ppm pertain to the two single protons H-7 and H-6 in the aromatic ring. They show a typical value for an aromatic ortho-coupling ($^3J = 7.7$ Hz). The two methyl groups result in triplets at $\delta = 0.72$ ppm (H-2″) and 1.24 ppm (H-2′), whereas the corresponding methylene groups are resolved as quartets at $\delta = 2.09$ ppm (H-1″) and $\delta = 2.75$ ppm (H-1′). The methylene group (H-3) near to the oxygen in the tetrahydropyrane ring shows a triplet at $\delta = 3.99$ ppm The other methylene groups (H-4, H-1‴) result in a multiplet at about 2.96 ppm The two very strong signals at 3.30 and 4.88 ppm belong to methanol ($CH\underline{H}D_2OD$) and water from the solvent.

The proof of the hydroxylation at position 5 was finally achieved by a ^{1}H-COSY 45 long-range experiment (Fig. 7). The arrow in the COSY spectrum shows the signal that results from the long-range coupling from the aromatic proton H-7 with the methylene protons of the ethyl group at position 8. This effect is only possible if etodolac is hydroxylated in position 5.

Because of the very low concentration of the analyte in the ^{13}C-NMR spectrum (not shown here), only the signals for primary, secondary and tertiary C atoms are observed. The signal assignment is as follows: ^{13}C-NMR (CD_3OD): δ (ppm) = 8.1 (CH_3, C-2″), 14.9 (CH_3, C-2′), 62.1 (CH_2, C-3), 104.6 (CH, C-6), and 121.4 (CH, C-7). The signals for the other CH_2 groups appear at 24.6, 25.3, 32.3 and 44.3 ppm

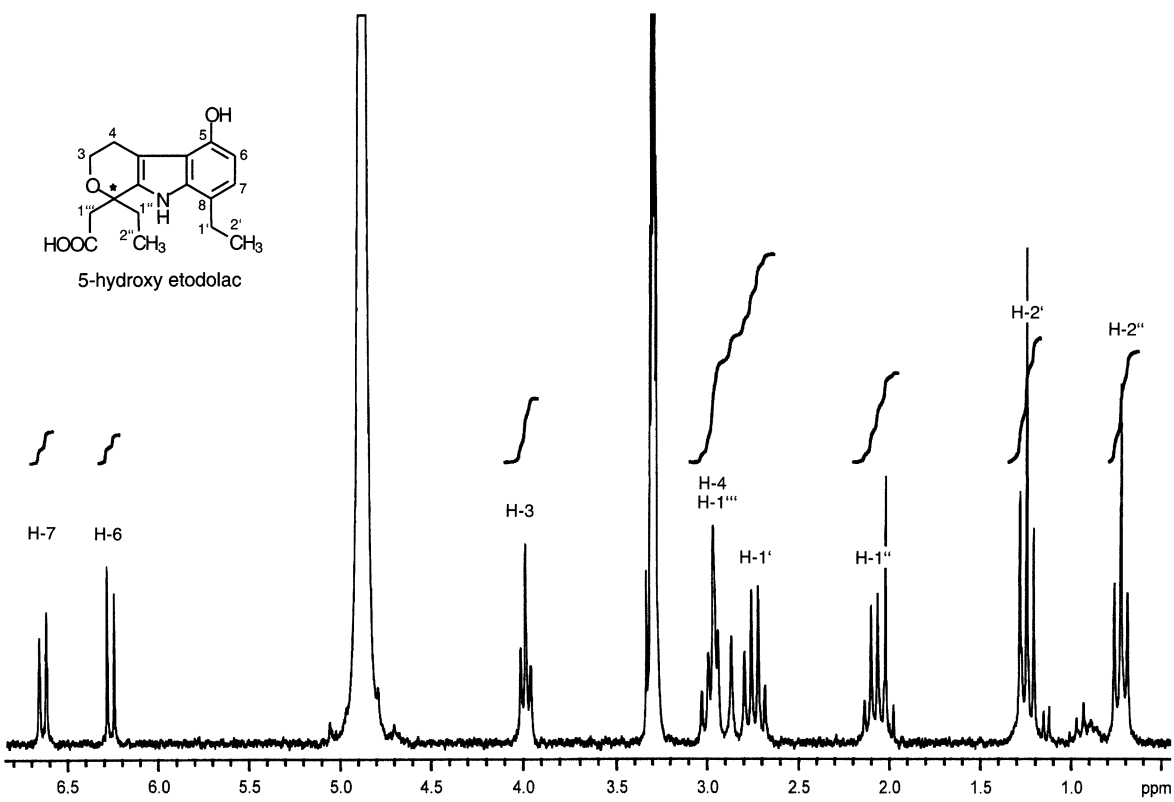

Fig. 6. ^{1}H-NMR spectrum (200 MHz) of metabolite X (5-hydroxy etodolac) in methanol-d_4.

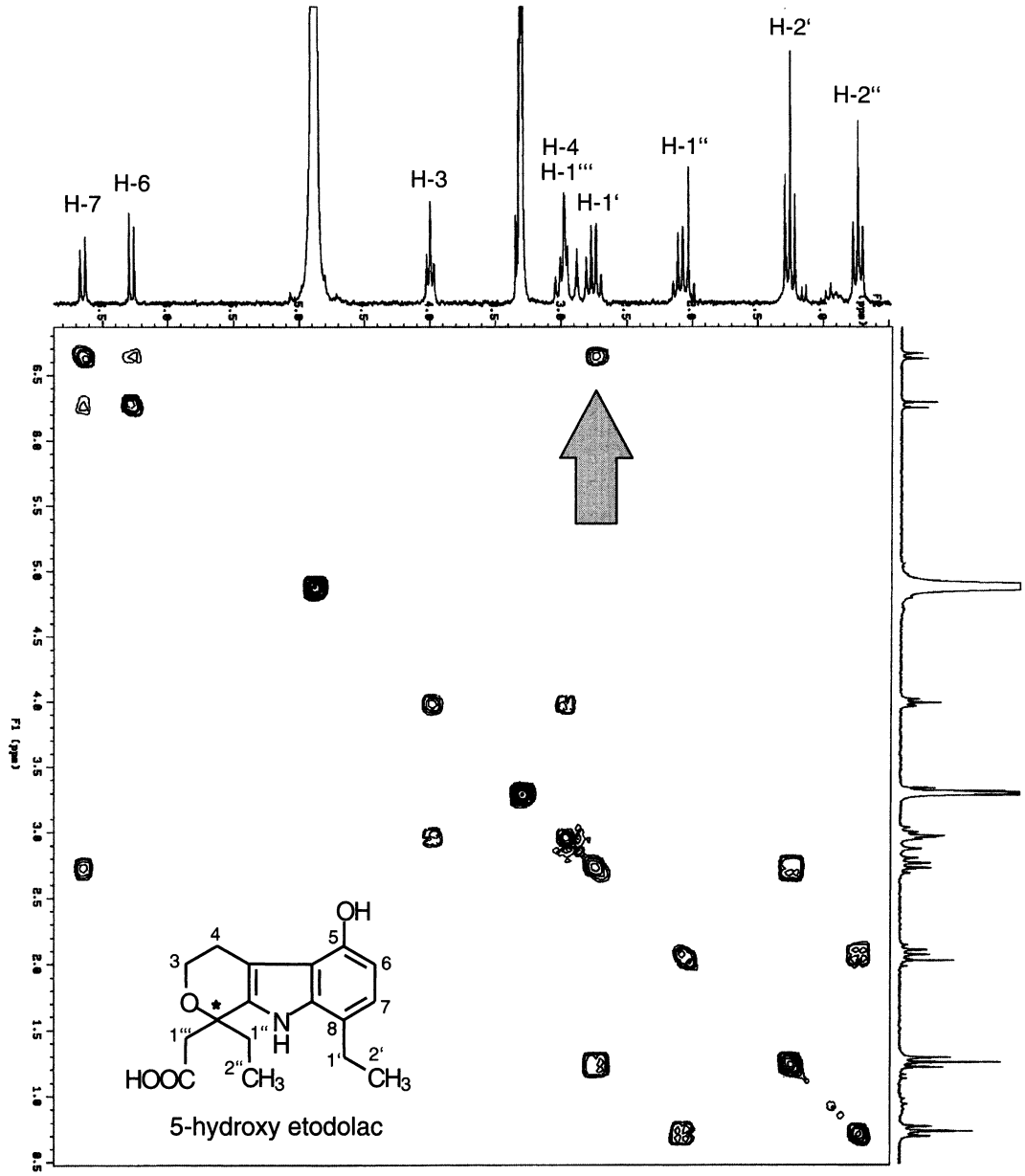

Fig. 7. ^{1}H-COSY 45 long-range spectrum (200 MHz) of metabolite X (5-hydroxy etodolac) in methanol-d_4.

4. Conclusions

In this investigation, it was possible to isolate an unknown monohydroxylated phase I metabolite (metabolite X) of the non-steroidal anti-inflammatory drug etodolac and identify it as 5-hydroxy etodolac by mass spectrometry and NMR experiments.

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