Non-invasive Detection of Ibuprofen *in Vivo* ^{13}C-NMR Signals in Rats

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A non-invasive *in vivo* ^{13}C-NMR experiment is performed to follow the metabolic pathway of ibuprofen in rats. Detection of possible intermediates and the stereoselectivity of ibuprofen chiral inversion process are discussed.

Key words ibuprofen metabolism; *in vivo* ^{13}C-NMR; xenobiotics

Ibuprofen, a nonsteroidal anti-inflammatory drug, can inhibit the function of cyclo-oxygenase in the synthesis of prostaglandins which participate in renal physiological processes.[1-4] Past studies have shown that the anti-inflammatory activity ratio of ibuprofen enantiomers, *S*(+)/*R*(−), is about 160 *in vitro*.[5] However, *R*-ibuprofen undergoes epimeric inversion to become its active *S* antipode *in vivo* and exhibits as high as 60% of the *S*-ibuprofen potency in mice, rats, guinea-pigs,[5,6] as well as in humans.[7,8] Ibuprofen metabolic studies have been conducted mainly by invasive means with the aid of NMR,[9,10] liquid chromatography,[11-15] and MS.[11,16] The aim of our work is to demonstrate the feasibility of using ^{13}C enriched ibuprofen to study ibuprofen metabolism *in vivo* by NMR methods and the possibility to study its stereoselectivity-an important property of the enzymatic metabolism of the xenobiotics.[17]

Figure 1 shows a generally accepted ibuprofen inversion mechanism proposed by Nakamura *et al.*[18] The chemical environment of the asymmetric center, C2, changes during the process. Thus the metabolism of each isomer, with ^{13}C enriched C2, can be traced by following the ^{13}C-NMR signals and chemical shift changes.

Experimental

R(−) and *S*(+) forms of [2-^{13}C,2-^{2}H]ibuprofen and their CoA thioesters with optical purity >98% enantiomeric excess and isotopically enriched were synthesized, purified, and separated as reported.[19,20]

A 4.7 T imaging system with 33 cm bore (Varian, Palo Alto, CA) was used to allow accommodation of life rats. ^{13}C-NMR spectra of *R*- and *S*-[2-^{13}C,2-^{2}H]ibuprofen, *R*- and *S*-[2-^{13}C]ibuprofen dissolved in sesame oil, and *R*-[2-^{13}C,2-^{2}H]ibuprofen-CoA thioester (aq.), with both ^{13}C and ^{2}H 99.9% enriched at C2, were obtained with $CDCl_3$ external reference, centered at 77.0 ppm.

Healthy adult rats (Sprague Dawley, 250—390 g) were used for *in vivo* studies. They were fed *ad libitum* with lab chow and had free access to water. The same 4.7 T imaging system was used. A five-turn solenoidal (4.5 cm i.d., 6 cm length) ^{13}C coil encircled the abdominal region and the liver so that the initial ibuprofen injected in the abdominal cavity and any metabolites which appeared mainly in the liver can be detected simultaneously. The rat was anesthetized with i.p. injection of Ketamine (80 mg/kg) and Rompun (12 mg/kg), and put at the center of the magnet in prone position. Proton signal from the same coil was used to shim the field. The line width of the water proton from the animal body was about 85 Hz (0.43 ppm). A background spectrum was taken initially, followed by remote i.p. injection of 100—130 mg of *R*-[2-^{13}C,2-^{2}H]-ibuprofen in 1 ml sesame oil. Thereafter, ^{13}C spectra were taken continuously and summed every 5-min interval for a total of 2—4 h. Acquisition parameters: resonance frequency 50.31 MHz, pulse length 100 μs (30—45° flip angle), repetition time 0.6—0.7 s, 10 kHz bandwidth, 2048 complex points of flame ionization detector (FID) data, $CDCl_3$ external reference, FT with 10 Hz Lorentzian line broadening. The same procedure was employed for the *S*-[2-^{13}C,2-^{2}H]ibuprofen.

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Results

For *in vitro* NMR studies, the ^{13}C chemical shifts obtained for the C2 of *R*- and *S*-[2-^{13}C,2-^{2}H]ibuprofen, and *R*- and *S*-[2-^{13}C]ibuprofen (the converted product), dissolved in sesame oil, were all around 44.3±0.3 ppm (not shown). The chemical shifts of them in aqueous buffered solution were around 42.6 ppm. Those of the *R*- and *S*-[2-^{13}C,2-^{2}H]ibuprofen-CoA in bufferred aqueous solution were around 53.4 ppm, no observation was made in sesame oil due to low solubility.

For *in vivo* NMR studies, the background spectrum taken from the abdominal region of the rat is shown in Fig. 2a, with no significant peaks in the 40—58 ppm region. When sesame oil alone was injected into the rat abdomen, similar spectrum was observed, and the spectrum did not change for three hours. No peaks were found in the 40—58 ppm region examined under magnified scales.

When *R*-[2-^{13}C,2-^{2}H]ibuprofen dissolved in sesame oil was injected, a peak at the 44.2 (+3) ppm appeared immediately (Fig. 2b), away from lipid background signals. The difference spectra, obtained by subtracting lipid

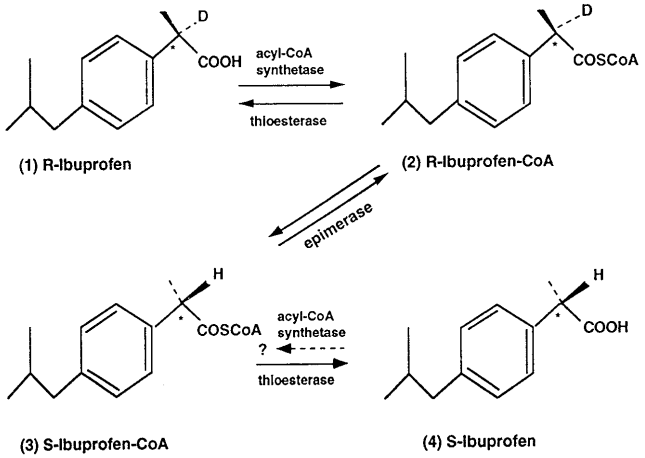

Fig. 1. A Schematic of the Conversion Process of *R*-Ibuprofen (**1**) to *S*-Ibuprofen (**4**)

2 and **3** are intermediates. The asymmetric C2 carbon is marked by an asterisk. In the presence of epimerase the deuterium is extracted and replaced by a proton nearby, with configuration of C2 inverted. The C2 chemical shift difference between **1** and **2** is detectable. Whether **4**→**3** occurs, is under study here.

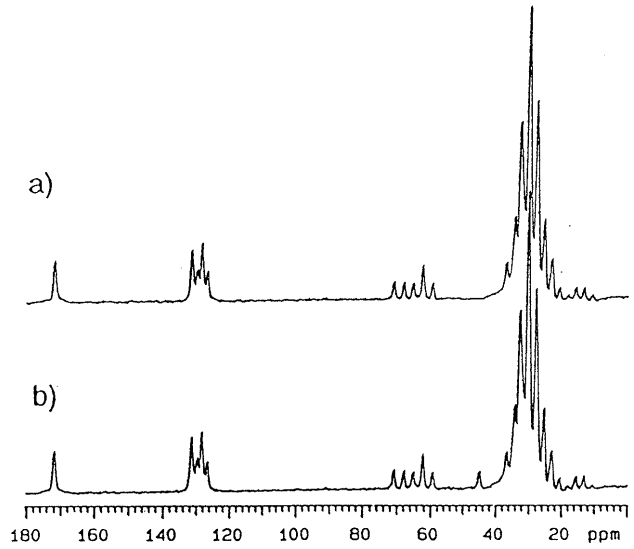

Fig. 2. a) Background ^{13}C-NMR Spectrum Taken from a Rat's Abdomen

Assignments: CH_2 groups at far right; glycerol backbone, 58—72 ppm; double bonds, 130 ppm; R-OCOR ester groups, 172 ppm. Sesame oil in rat exhibits this same spectrum.

b) Additional Peak Appeared When *R*-[2-^{13}C, 2-^{2}H]Ibuprofen, Dissolved in Sesame Oil, was Injected

Other ibuprofens appeared around the same location. For imaging systems, the field homogeneity is compromised to achieve a larger volume of interest. Thus signal multiplets, resulting from the coupling of carbon to deuterium, are collapsed into their individual singlets as shown.

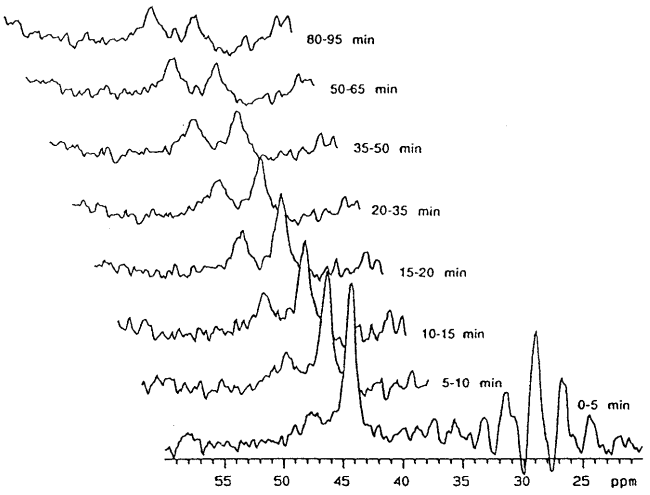

Fig. 3. Difference ^{13}C Spectra, Obtained by Subtracting Background Lipid Signals (Fig. 2a) from Spectra Accumulated at Every 5-min Interval after i.p. Injection of *R*-[2-^{13}C, 2-^{2}H]Ibuprofen into the Rat

Only the residual lipid signals, from 20 to 36 ppm, of the first spectrum are shown here for clarity.

spectrum from spectra after ibuprofen injection, are presented in Fig. 3. The 44.2 ppm peak diminished with time. A peak at 48.1 ppm emerged and was detectable from the first 5-min onward. Three rats were done. The time-course of the peaks were not identical among all rats, however, the location and the time-dependent intensity variation of the peaks were reproducible. After 2—3 h, only a small 44.2 ppm peak was present, the 48.1 ppm peak became undetectable.

Similar experiments were conducted for *S*-[2-^{13}C,2-^{2}H]ibuprofen on six rats. In seeking a balance between

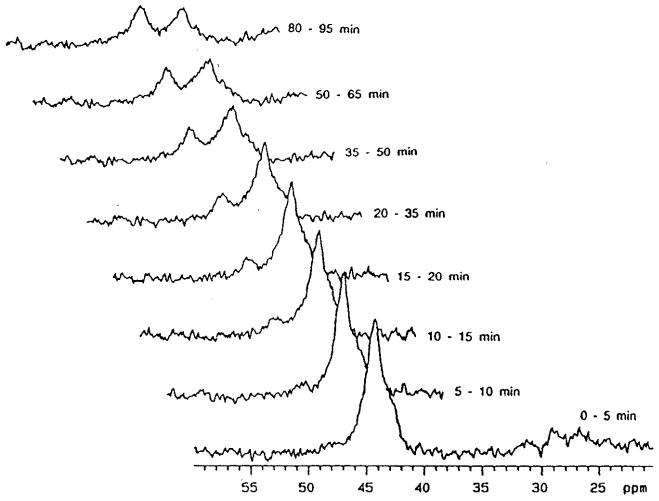

Fig. 4. Difference ^{13}C Spectra, Similarly Obtained as in Fig. 3, but with *S*-[2-^{13}C, 2-^{2}H]Ibuprofen (dose 200 mg) Injected

Note that the 48 ppm peak appeared at a later time as compared to the *R* substrate in Fig. 3.

minimum dosage and a detectable effect, we varied doses from 50 mg to 200 mg per rat. All spectra showed similar progression as those of the *R*-isomer. A peak at the 44.3 ppm was prominent in the first 5-min data collection. However, the second peak at 48.2 ppm became visible only 10 min after injection and under higher doses (150—200 mg). Figure 4 shows spectra with 200 mg *S*(+) administered.

Discussion

The deuterium enrichment was not utilized. We used it for consistency with our previous experiments. There is no deuterium isotopic effects.[11] The general progression of the relevant peaks was reproducible for both the *R*(−) and the *S*(+) isomers. Variation of metabolic rates among rats was present and was noted in other reports as well.[11] For this reason we did not combine our data sets. Proton decoupling was found ineffective in enhancing the signal due to rf power deficiency and possibly due to a tertiary C2. For sake of brevity and clarity in discussion, we denote in the following the peaks at or in the neighborhood of 44.2 ppm the 44 ppm peak, and those at or near 48.1 ppm the 48 ppm peak.

The 44 ppm peak is unambiguously assigned as the C2 of the *R*(−) or *S*(+)-ibuprofen peak as the chemical shift is the same both *in vitro* and *in vivo*. The decrease in intensity after injection is due to ibuprofen dissipation to other parts of the body. For time series studies, this peak represents the sum of the starting drug administered and the product, minus wash-aways.

The 48 ppm peak was assigned to metabolic intermediate(s). Its occurrence from sesame oil metabolism was ruled out as no peaks were found near 48 ppm from sesame oil injection in rat. Partitioning of ibuprofen into the aqueous phase was also eliminated because ibuprofens in aqueous solutions shifted to 42.6 ppm. Human urinary metabolite studies by HPLC and MS[21,22] and deuterium-labeled studies in rats,[16,20,23] showed that ibuprofen metabolism proceeds mainly by: 1) esterification and hydrolysis at the acid terminal attached

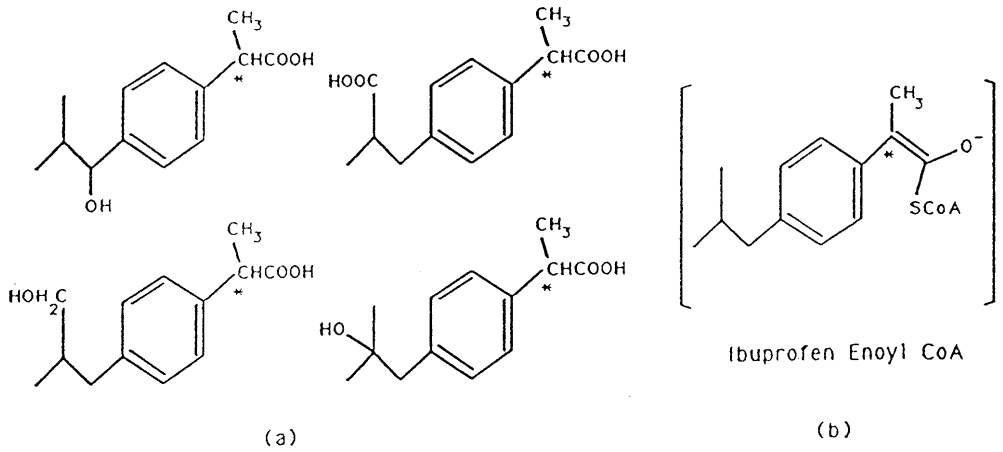

Fig. 5. (a) Confirmed Forms of Ibuprofen Metabolites[11,21]

The isobutyl chain, far away from C2 of the phenyl ring, is oxidized. Thus, the oxidative reaction will not affect the chemical shift of the C2 atom significantly. C2 does not sever from the ibuprofen molecule during conversion, metabolism or after disposition.

(b) Ibuprofen Enoyl CoA Intermediate, Proposed by Sanins *et al.*,[16] with a Planar sp^2 Configuration

This species, if exists, should be present between steps **2** and **3** of Fig. 1.

to the C2 position, giving off ibuprofen itself; and 2) oxidative reactions. For either case, it has been verified that there is no degradation of the ibuprofen molecule into smaller fragments during the metabolism or after its disposition. For case 1), only a deuterium was lost (exchanged by a proton, causing a 0.5 ppm shift[10] undetectable by imaging machine) from C2 with concomitant chirality inversion, no C2–C bonds were severed. Thus, the ^{13}C2 stays with ibuprofen all the time. For most oxidized ibuprofens, an H at the *para*-isobutyl chain is replaced by an OH, or further oxidized to an acid (Fig. 5a).[21] Note that these oxidations, occurring at carbons far away from C2, will not affect the ^{13}C2 chemical shift appreciably, *i.e.*, ibuprofen metabolites have the same ^{13}C2 chemical shift (44 ppm) as ibuprofen itself. In summary, since the ^{13}C2 label (the only thing we followed) stays on the ibuprofen all the time, then the 48 ppm ^{13}C peak, belonging neither to ibuprofen nor to any of its possible metabolites, must come from intermediates produced in between.

As shown in Fig. 1, ibuprofen attaches to CoA factor with the aid of a synthetase, and then undergoes inversion by the participation of an epimerase. At either stage, such an entity, large and immobile, usually possess very broad NMR signals and escapes detection. Therefore, the visible 48 ppm signal should have arisen from a smaller entity. Without further detailed study, we can only speculate at this moment that it is more likely to be the intermediate anion, ibuprofen-Enoyl-CoA, with C2 at a planar sp^2 carbanion state as proposed by Sanins *et al.*[16] (Fig. 5b). The 48 ppm peak is 4 ppm downfield from the parent ibuprofen peak. It is consistent with previous *in vitro* study of [2-^{13}C]ibuprofen-CoA relative to [2-^{13}C]ibuprofen (with/without 2-^{2}H)[10] where a downfied value of 5 ppm was observed. This slight difference might be an indication that the C2 of the intermediate we detected is not in tetrahedral form as in synthesized ibuprofen-CoA, but in some other form such as planar as just stated. Other sources of discrepancy might have come from magnetic susceptibility effects, common in NMR imaging systems. they originate from factors such as: field direction differences[24]; external reference; internal organ interfaces; and possible trace amount of paramagnetic materials.

There were some studies done on ibuprofen stereospecificity.[13,20,25,26] In our study, when $S(+)$ was injected, a delayed 48 ppm intermediate peak did occur, consistent with partial conversion of $S(+)$ to $R(-)$ through the same intermediate as reported by others.[11,17,26,27] It also indicates that the inversion is not stereospecific, but is stereoselective of R over S. When lower $S(+)$ doses were used, no significant 48 ppm peak could be detected. However, when 200 mg $S(+)$ was injected, the 48 ppm peak area for the 80—95 min trace was abut 20% of the initial peak (44 ppm) area (Fig. 4). This fraction is too high to be considered as optical isomer contamination from preparation (*ca.* 0.1%). Similar dosage dependency was reported by Fournel and Caldwell.[25]

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