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Determination of ibuprofen in dog plasma by liquid chromatography and application in pharmacokinetic studies of an ibuprofen prodrug in dogs

Peng Wang [a,c], Meiling Qi [b,*], Lihe Liu [c], Lin Fang [a]

[a] *Shenyang Pharmtech Institute of Pharmaceuticals, Shenyang 110016, China*
[b] *Department of Chemistry, School of Science, Beijing Institute of Technology, 5 South Street, Zhong Guan Cun, Beijing 100081, China*
[c] *School of Pharmaceutical Engineering, Shenyang Pharmaceutical University, Shenyang 110016, China*

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Abstract

A liquid chromatography (LC) method for the determination of ibuprofen in dog plasma is described. Chromatographic separation was performed on a Diamonsil™ C_{18} column with a C_{18} guard column using a binary mixture of acetonitrile and 0.02 mol/l phosphate buffer (pH 6.5) (35:65, v/v) delivered at a flow rate of 1.2 ml/min. The linear range for ibuprofen was from 1.0 to 40.0 μg/ml with a limit of quantitation of 1.0 μg/ml. Within-run accuracy and precision ranged from −0.1% to 4.0% and from 1.1% to 5.5% and between-run accuracy and precision ranged from −1.1% to 4.7% and from 1.3% to 7.0%, respectively. The mean extraction recoveries of ibuprofen determined over the concentrations of 1.0, 10.0, and 40.0 μg/ml were (100.5 ± 1.8)%, (99.8 ± 1.0)%, and (99.2 ± 2.3)%. The developed LC method greatly simplified the sample preparation and adopted mild conditions to prevent the possible hydrolysis of the prodrug and was successfully applied to the pharmacokinetic studies of an ibuprofen prodrug in dogs.
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Keywords: Ibuprofen; Prodrug; Liquid chromatography; Dog plasma

1. Introduction

Ibuprofen, a non-steroidal anti-inflammatory drug (NSAID), exerts significant anti-inflammatory, analgetic, and anti-pyretic effects in clinical trials. But like other NSAIDs, it also causes severe gastrointestinal side effects including gastric distress and peptic ulcers, which possibly result from two different mechanisms, a direct contact effect and a generalized systemic effect. The first side effect is cased by the combination of local irritation produced by the carboxylic group in the molecular structure and local inhibition of prostaglandin synthesis in the gastrointestinal tract. It is has been demonstrated that prodrug approach by means of the esterification of carboxylic group in the structure is a promising way of overcoming gastric injury associated with long term oral use of NSAIDs like ibuprofen [1–3].

Ligustrazine (tetramethylpyrazine) is a biologically active ingredient isolated from the traditional herbal medicine *Ligusticum chuanxiong* Hort. Ligustrazine possesses anti-ulcer and anti-platelet activities and has been widely used in China for the treatment of patients with angina pectoris, ischemic vascular diseases and gastric lesion [4–6]. Hydroxyl ligustrazine is one active metabolite of ligustrazine and exhibiting similar effects with ligustrazine. The hydroxyl group in the structure can serve as a handle for the prodrug formation of ibuprofen with hydroxyl ligustrazine.

Ibuprofen ligustrazinate hydrochloride (Fig. 1), a prodrug of ibuprofen with hydroxyl ligustrazine, was developed in our laboratory to suppress the gastric injury of ibuprofen and improve its anti-platelet activities. A prodrug is often targeted to biologically transform to its parent drugs after oral administration. In the development of this prodrug, a biopharmaceutical test in dogs should be made to demon-

* Corresponding author. Tel.: +86 10 68912667; fax: +86 10 68913293.
E-mail address: mlqi@bit.edu.cn (M. Qi).

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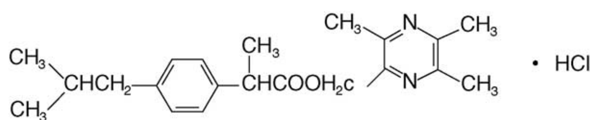

Fig. 1. Structure of ibuprofen ligustrizinate hydrochloride.

strate if ibuprofen could be rapidly released from the prodrug after oral administration. To meet this need, an analytical method for the determination of ibuprofen in dog plasma was required.

Liquid chromatography (LC) with ultroviolet detection is often preferred in ordinary laboratories for its low cost, wide availability and high precision and accuracy. Several LC methods with UV detection are available for the determination of ibuprofen in biological fluids [7–13]. Almost all the LC methods published for the determination of ibuprofen in plasma were performed in acidic conditions with a pH value ranging from 2 to 4, which were not suitable for our work. To prevent the prodrug from hydrolytic decomposition during sample preparation and chromatographic analyses, acidic or basic conditions should be avoided in our work.

The present paper describes a simple and selective LC method for the determination of ibuprofen in dog plasma. In this work, a simple procedure combining deproteinization and extraction into a single process was taken for the sample preparation. The described LC method greatly simplified the sample preparation and adopted mild conditions for both sample preparation and chromatographic separation to minimize the possible hydrolysis of the prodrug and was successfully applied to the pharmacokinetic studies of a prodrug of ibuprofen with hydroxyl ligustrizine.

2. Experimental

2.1. Reagents and chemicals

Ibuprofen reference standard (99.8% purity) was from Shenyang Shengyuan Pharmaceutical Company (Shenyang, China) and naproxen reference standard (99.8% purity) was from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Ibuprofen ligustrazinate hydrochloride reference standard and capsules (each capsule equivalent to 100 mg ibuprofen) were from Shenyang Pharmtech Institute of Pharmaceuticals (Shenyang, China). Ibuprofen capsules (each capsule containing 100 mg ibuprofen) used as reference preparation were a commercial product from Shandong Xinhua Pharmaceutical Company Ltd. (Zhibo, Shandong). LC-grade methanol and acetonitrile were purchased from Tianjin Concord Science and Technology Company Ltd. (Tianjin, China). Sodium hydroxide and potassium dihydrogen phosphate were analytical-grade chemicals from Shenyang Reagent Company (Shenyang, China).

2.2. Instrumentation

Chromatographic separation was performed with an HP series 1100 chromatographic system equipped with a G1310A isocratic pump, a G1314A variable UV–vis detector, a 3395 integrator and a G1328A manual injector with a 20 μl loop (Agilent, USA). A Shimadzu UV-2201 double-beam spectrophotometer (Shimadzu, Japan) was used for scanning and selecting the detection wavelength.

2.3. Preparation of calibration standards and quality control samples

Stock solutions of ibuprofen and naproxen (internal standard) were individually prepared at 1 mg/ml in methanol and stored at 4 °C. The stock solution of ibuprofen was further diluted with methanol to give a series of standard solutions with concentrations of 10, 20, 50, 100, 200, and 400 μg/ml. A diluted solution containing 10 μg /ml of naproxen in methanol was also prepared.

Calibration standards of ibuprofen (1.0, 2.0, 5.0, 10.0, 20.0, and 40.0 μg/ml) were prepared by spiking appropriate amount of the standard solutions in blank plasma obtained from healthy and non-smoking volunteers. Quality control (QC) samples were prepared using the pooled plasma at concentrations of 1.0, 10.0, and 40.0 μg/ml. The spiked samples were then treated following the sample preparation procedure as indicated in Section 2.4.

2.4. Sample preparation

To 0.5 ml of dog plasma, 50 μl of methanol, 100 μl of naproxen solution (10 μg/ml), and 1.5 ml of methanol were added and vortex mixed for 5 min and centrifuged for 5 min. The separated supernatant was evaporated to dryness in a water bath at 50 °C under the protection of nitrogen. The residue was reconstituted with 200 μl of mobile phase and 20 μl was injected onto the LC system.

2.5. Chromatographic conditions

An analytical column, Diamonsil ™ C_{18} column (150 mm × 5.0 mm, 5 μm) from Dikma Technologies (Beijing, China) and a DL-II type guard column packed with YWG-C_{18} (10 mm × 4.0 mm, 10 μm) from Tianjin Chromatographic Science and Technology Company (Tianjin, China) were used for chromatographic separation. Mobile phase consisted of a binary mixture of acetonitrile and 0.02 mol/l potassium dihydrogen phosphate buffer adjusted to pH 6.5 with 10% sodium hydroxide solution (35:65, v/v) delivered at a flow rate of 1.2 ml/min. The detection was made at 223 nm. Chromatography was performed at room temperature and the run time was less than 15 min.

2.6. Method validation

Validation runs were conducted on three separate days. Each validation run consisted of a set of the spiked stan-

dard samples at six concentrations over the concentration range (each in triplicate) and QC samples at three concentrations (1.0, 10.0, and 40.0μg/ml, $n = 6$ at each concentration). Standard samples were analyzed at the beginning of each validation run and other samples were distributed randomly throughout the run. The results from QC samples in three runs were used to evaluate the accuracy and precision of the method developed. Concentrations of ibuprofen in plasma samples were determined by back-calculation of the observed peak area ratios of the analyte and internal standard from the best-fit calibration curve using a weighted ($1/x^2$) linear regression. During routine analysis, each analytical run included a set of standard samples, a set of QC samples in duplicate and plasma samples to be determined.

The extraction recovery of ibuprofen was determined at low, medium and high concentrations ($n = 3$ at each concentration) by comparing the area ratio of ibuprofen to internal standard from plasma samples spiked before extraction with those from plasma samples spiked after extraction.

Sample stability in plasma stored at room temperature for 24 h and at −20 °C for 2 months was determined by analyzing QC samples at concentrations of 1.0, 10.0, and 40.0 μg/ml in duplicate.

2.7. Application of the developed LC method

The LC method developed was successfully applied to the pharmacokinetic studies of ibuprofen ligustrazinate hydrochloride capsules in dogs. Six dogs (10–15 kg) were from the Laboratory Animal Center of Shenyang Pharmaceutical University. This study was based on a single-dose, randomized, two-treatment, two-period crossover design. In the morning of phase I, after an overnight fast (10 h), six dogs were given single dose of either two test capsules (a dose equivalent to 200 mg ibuprofen) or two reference capsules (a dose equivalent to 200 mg ibuprofen) which were contained in small amount of sausage. No other food was allowed until 4 h after dose administration while water intake was free. About 2 ml of blood samples was collected from the foreleg vein into heparinized test tubes before (0 h) and at 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8, 10, 12, and 24 h after dosing. Plasma was separated by centrifugation and kept frozen at −20 °C until analysis. After a washout period of 7 days, the study was repeated in the same manner to complete the crossover design.

3. Results and discussion

3.1. Development of the LC method

To determine ibuprofen released from the prodrug in plasma, the possible conversion of the prodrug into the parent drugs should be minimized during both the sample preparation and chromatographic analysis. In both processes, acidic or basic conditions should be avoided to prevent the prodrug from hydrolytic decomposition.

In this work, a simple procedure was taken for the sample preparation. Methanol was used both as a deproteinizing agent and as an extracting agent. The extraction recoveries for both ibuprofen and internal standard were satisfactory. This procedure combined the deproteinization and extraction into a single process, which greatly simplified the sample preparation and minimized the possible hydrolysis of the prodrug.

In the development of chromatographic conditions, a C_{18} column is often the first choice for most of the chromatographic analyses of drugs. So did in this work and it worked. Besides the column, the composition of mobile phase is another key factor among the chromatographic conditions. Almost all the mobile phases in the LC methods published for the determination of ibuprofen in plasma were performed in acidic conditions with a pH value ranging from 2 to 4, which were not suitable for our work. A mobile phase with a neutral pH value was preferred in this work. Based on this requirement, acetonitrile and phosphate buffer became our choice. The type and molar concentration of phosphate buffer and the ratio between acetonitrile and phosphate buffer were optimized through several trials to achieve good resolution and symmetric peak shapes of analytes as well as short run time. It was found that a mixture of acetonitrile and 0.02 mol/l potassium dihydrogen phosphate buffer adjusted to pH 6.5 with 10% sodium hydroxide solution (35:65, v/v) delivered at a flow rate of 1.2 ml/min with detection at 223 nm could achieve our purpose and was finally adopted. Moreover, it was necessary to reconstitute the residues with the mobile phase to produce the expected peak shapes of the analytes.

3.2. Selectivity

The method selectivity was evaluated by analysis of dog plasma samples. Typical chromatograms are shown in Fig. 2. Ibuprofen and naproxen exhibited retention times of 11.5 and 4.8 min, respectively. Baseline resolution was achieved without interference of endogenous substances from the blank dog plasma. Ibuprofen can also be well resolved from the prodrug with the described LC method (Fig. 3). The retention time for the prodrug was 3.8 min. The results show that the described LC method is selective for the determination of ibuprofen in dog plasma.

3.3. Linearity

The linearity of each calibration curve was determined by plotting the peak–area ratio (y) of ibuprofen to internal standard versus the nominal concentration (x) of ibuprofen. The calibration curves were obtained by weighted ($1/x^2$) linear regression analysis. To evaluate the linearity of the developed method, plasma calibration curves were determined in triplicate on three separate days. Representative regression equa-

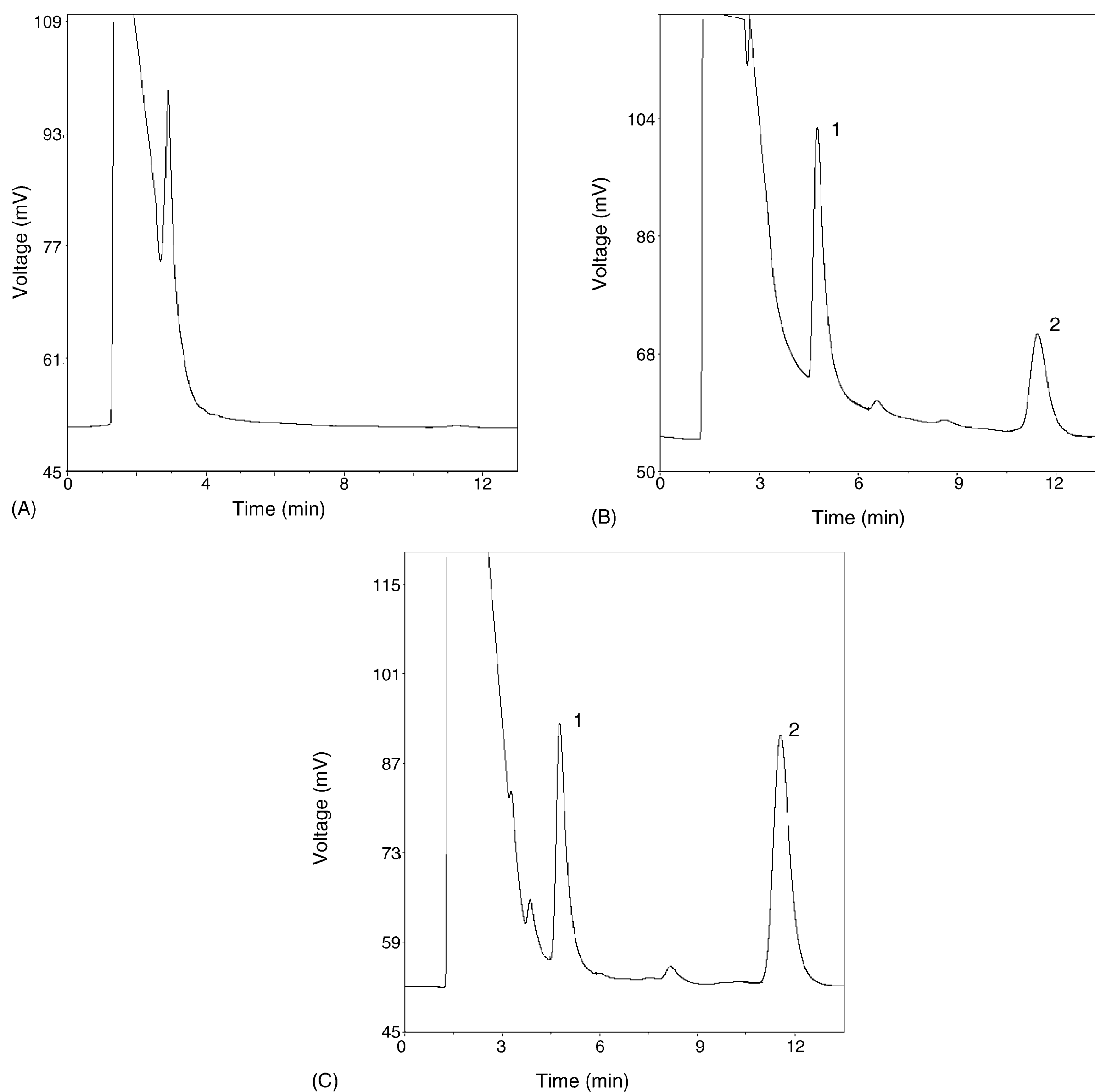

Fig. 2. LC chromatograms for the method selectivity: (A) blank dog plasma; (B) blank plasma spiked with 5.0 μg/ml ibuprofen and 10 μg/ml internal standard; (C) a dog plasma sample collected from foreleg vein at 3 h after oral administration of ibuprofen prodrug capsules. Peaks: (1) naproxen ($t_R = 4.8$ min) and (2) ibuprofen ($t_R = 11.5$ min).

tion for the calibration curve was $y = 7.07 \times 10^{-3} + 0.121x$ with a correlation coefficient of 0.9998. Good linearity was observed over the concentration range of 1.0–40.0 μg/ml for ibuprofen.

3.4. Limit of quantitation

The lower limit of quantitation (LLOQ) for determination of ibuprofen in dog plasma, defined as the lowest concentration above which quantitation can be carried out with adequate accuracy and precision (Table 1), was found to be 1.0 μg/ml, which is sufficient for pharmacokinetic studies of ibuprofen preparations in dogs.

3.5. Accuracy and precision

The accuracy and precision of the present method were determined using dog plasma samples freshly prepared at ibuprofen concentrations of 1.0, 10.0, and 40.0 μg/ml. Six replicate determinations were made for each concentration and relative standard deviation (R.S.D.) served as measure of the precision. The accuracy was determined by comparing the measured concentrations with the expected concentrations of ibuprofen in spiked blank dog plasma and expressed as relative error (RE). As shown in Table 1, the within-run precision and accuracy of the method are less than 5.5% and 4.0%, respectively, and the between-run precision and accu-

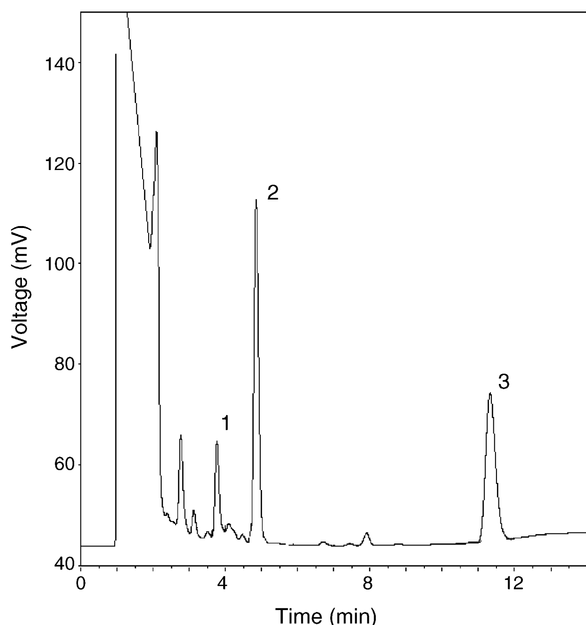

Fig. 3. LC chromatogram of a mixture of (1) ibuprofen prodrug (t_R = 3.8 min), (2) naproxen (t_R = 4.8 min), and (3) ibuprofen (t_R = 11.5 min).

Table 1
Accuracy and precision of the developed LC method for the determination of ibuprofen in dog plasma

	Concentration (μg/ml)		
	1.0	10.0	40.0
Within-run (n = 6)			
Mean ± S.D. (μg/ml)	1.04 ± 0.06	10.0 ± 0.11	39.96 ± 1.13
R.S.D. (%)	5.5	1.1	2.8
RE (%)	4.0	0.0	−0.1
Between-run (n = 6)			
Mean ± S.D. (μg/ml)	1.05 ± 0.07	10.19 ± 0.13	39.55 ± 0.76
R.S.D. (%)	7.0	1.3	1.9
RE (%)	4.7	1.9	−1.1

S.D.: standard deviation; R.S.D.: relative standard deviation; RE: relative error. RE (%) = 100 × ((mean concentration − nominal concentration)/nominal concentration).

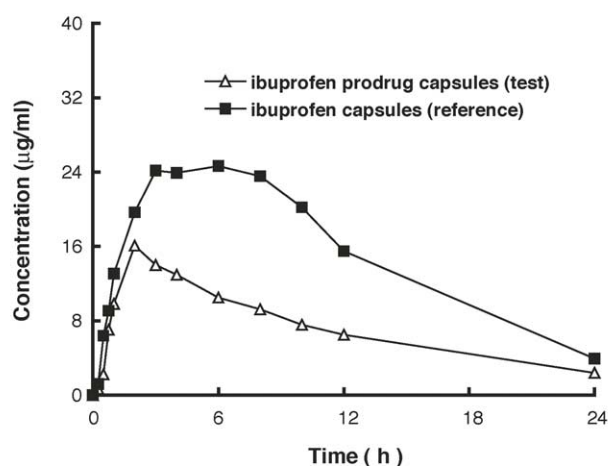

Fig. 4. Mean plasma concentration–time profiles of ibuprofen in six dogs after oral administration of ibuprofen prodrug capsules (a dose equivalent to 200 mg ibuprofen) and ibuprofen capsules (200 mg ibuprofen) to dogs.

racy are less than 7.0% and 4.7%. The results demonstrate the acceptable accuracy and precision of the developed LC method.

3.6. Extraction recovery

The mean extraction recoveries of ibuprofen determined over the concentrations of 1.0, 10.0, and 40.0 μg/ml were (100.5 ± 1.8)%, (99.8 ± 1.0)%, and (99.2 ± 2.3)%. For the internal standard (10 μg/ml), the mean extraction recovery was (100.9 ± 1.0)%.

3.7. Stability

The stability of ibuprofen in plasma was evaluated by analyzing quality control samples (1.0, 10.0, and 40.0 μg/ml) stored at room temperature for 24 h and at −20 °C for 2 months, respectively. As shown in Table 2, the mean concen-

Table 2
Stability of ibuprofen in dog plasma after 24 h at 20°C and 2 months after stored at −20 °C

	Concentration (μg/ml)					
	1.0		10.0		40.0	
	1[a]	2[a]	1[a]	2[a]	1[a]	2[a]
24 h at 20 °C						
Found (μg/ml)	1.01	0.99	10.07	10.11	39.33	38.67
Mean (μg/ml)	1.00		10.09		39.00	
Recovery (%)	100.0		100.9		97.5	
Mean ± S.D. (%)			99.5 ± 1.8			
2 months at −20 °C						
Found (μg/ml)	0.96	0.98	9.90	9.85	38.41	38.56
Mean (μg/ml)	0.97		9.88		38.48	
Recovery (%)	97.0		98.8		96.2	
Mean ± S.D. (%)			97.3 ± 1.3			

S.D.: standard deviation.
[a] Numbers.

trations of ibuprofen in plasma did not change significantly within the time period under the indicated store conditions.

3.8. Application of the developed LC method

The developed LC method has been successfully used for the pharmacokinetic studies of ibuprofen ligustrazinate hydrochloride capsules (test preparation) and ibuprofen capsules (reference preparation) in dogs following oral administration. The mean plasma concentration–time profiles for the two formulations are shown in Fig. 4.

4. Conclusions

The present LC method is simple and selective for the determination of ibuprofen in dog plasma. This method greatly simplified the process of sample preparation and adopted mild conditions to minimize the possible hydrolysis of the prodrug. It can be used for the pharmacokinetic studies of ibuprofen preparations including ibuprofen prodrugs.

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