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Determination of lefucoxib in rat plasma, urine, and feces by high-performance liquid chromatography with fluorescence detection: Application in pharmacokinetic studies

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Abstract

A sensitive, specific, and reproducible high-performance liquid chromatography (HPLC) method with fluorescence detection was developed for determination of lefucoxib in rat plasma, urine, and feces. The method involved liquid–liquid extraction using methyl *tert*-butyl ether, and celecoxib was used as the internal standard. The chromatographic separation was performed on a Kromasil C_{18} column (250.0 mm × 4.6 mm, 5.0 μm) with a mobile phase gradient consisting of water and methanol at a flow rate of 1 ml min^{-1}. The assay was linear in the range of 5.0–1000.0 ng ml^{-1} with a correlation coefficient (r) of 0.9994. The limit of quantification was 5.0 ng ml^{-1}. Inter- and intra-assay precisions were ≤14.2% and 5.5%, respectively. Relative recoveries ranged from 97.9% to 108.1%, and absolute recoveries were about 70.0% both with and without internal standard. All biological matrices (plasma, urine, and fecal homogenate) containing lefucoxib were stable for 5 h at room temperature (about 20 °C) and they are also stable after freeze–thaw cycles. The method was successfully applied to the pharmacokinetic studies of lefucoxib in rats.

Keywords: Lefucoxib; High-performance liquid chromatography; Fluorescence; Pharmacokinetic

1. Introduction

Non-steroidal antiinflammatory drugs (NSAIDs) have proven analgesic and anti-inflammatory properties, but also have significant gastrointestinal toxicity. The gastrointestinal toxicity appears to be related to COX-1 inhibition [1]. In 1990, Fu et al. [2] detected a novel COX protein in monocytes stimulated by interleukin, and a year later Kujubu et al. [3] identified a gene with considerable homology to COX-1. Identification of this COX-2 protein rekindled the effects of the pharmaceutical industry to produce a safer analgesic, anti-inflammatory drug via selective inhibition of COX-2. The COX-2 inhibitors generated rapid growth in the international antiarthritic market. Up to now, this class of drugs includes celecoxib, etoricoxib, valdecoxib, parecoxib, and other coxibs.

Lefucoxib (5-(3,4-dimethyl-phenyl)-1-methanesulfonyl-3-trifluoromethol-pyrazole) (Fig. 1) was one of new selective inhibitors of COX-2, and it showed potent anti-inflammatory and analgesic activity. The incidence of gastrointestinal complications caused by lefucoxib was significantly lower than that with the non-selective NSAIDs (the efficacy study will be detailed elsewhere later). To address the pharmacokinetics in preclinical species, it is essential to have a highly sensitive analytical method that can accurately measure low levels of lefucoxib in small volumes of plasma, urine, and feces. Several methods have been reported for the detection of COX-2 inhibitors in biological fluids, such as HPLC with ultraviolet (UV) detection [4–10], MS [11,12], and MS–MS [13]. To our knowledge, there is still no method reported for the determination of lefucoxib in biological matrices. The objective of the current effort was to develop a reproducible and sensitive HPLC fluorescence method to quantify lefucoxib in the large numbers of low-volume biological matrices generated in pharmacokinetic studies. We also demonstrated the applicability of this method for preclinical pharmacokinetic studies in rats.

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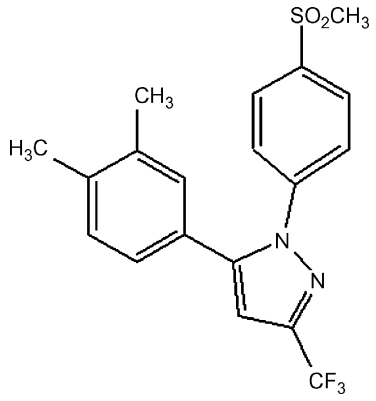

Fig. 1. The chemical structures of lefucoxib.

2. Materials and methods

2.1. Materials

Lefucoxib and celecoxib (internal standard, I.S.) were provided by China PLA General Hospital (Beijing, China). 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), and sodium hydroxide (analytical grade) was bought from Beijing Beihua Fine Chemicals Company, Limited (Beijing, China). The drug-free (blank) rats were provided by Laboratory Animal Center in Academy of Military Medical Science (Beijing, China).

2.2. Standard solutions

Stock solution of lefucoxib was prepared by dissolving 10 mg lefucoxib in 10 ml acetonitrile to obtain a concentration of 1 mg ml^{-1}. Then 0.1, 0.2, 0.4, 1.0, 2.0, 10.0, and 20.0 μg ml^{-1} working standards were prepared from the stock solution by dilution in acetonitrile and stored at +4 °C.

The solution of I.S. (1 mg ml^{-1}) was prepared in acetonitrile. The final solution was obtained by diluting this solution with acetonitrile to give concentration of 20.0 μg ml^{-1} and stored at +4 °C.

2.3. Preparation of calibration standards and quality control samples

Fecal samples were homogenized with water in the ratio 1:3 (100 g of feces with 300 g of water) to obtain fecal homogenate. To 0.19 ml of the blank rat plasma, urine, and fecal homogenate, 0.01 ml of above-mentioned lefucoxib working standards were added to yield final respective concentrations as 5.0, 10.0, 20.0, 50.0, 100.0, 500.0, and 1000.0 ng ml^{-1} of lefucoxib in plasma, urine, and fecal homogenate, respectively. Quality control (QC) samples (10.0, 500.0, and 1000.0 ng ml^{-1}) were prepared in a similar manner.

2.4. Sample preparation

0.2 ml of biological matrices (plasma, urine, and fecal homogenate) were added 10.0 μl I.S. solution (20.0 μg ml^{-1}), 50.0 μl sodium hydroxide solution (1 mol l^{-1}), and 500.0 μl methyl *tert*-butyl ether. The mixture was vortex mixed for 1 min and centrifuged at 10,000 × *g* for 5 min, and then the supernatant layer was removed. 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 dissolved in 80.0 μl of a mixed solution (methanol–water, 80:20, v/v) and 50.0 μl was applied to the HPLC apparatus.

2.5. Chromatographic conditions

The analysis of lefucoxib 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 (LC) systems.

Lefucoxib is very easy to dissolve in organic solution, such as methanol and acetonitrile, but it can hardly be dissolved in water. Hence 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 mobile phase consisting of methanol–water (80:20, v/v) for plasma samples, and for urine and feces samples a gradient elution was used to elute lefucoxib and I.S. from the column (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) at a flow rate of 1 ml min^{-1}. The temperature of the thermostated oven containing the column was set at 20 °C and the injection volume was 50 μl. Fluorescence detection was performed at an excitation wavelength of 254 nm and an emission wavelength of 430 nm.

2.6. Pharmacokinetic study

The pharmacokinetic study was carried out in five Wistar rats (male, weighing 250 ± 10 g). Before administration, rats had been fasted for 12 h but were allowed access to water throughout the experimental period. Animals were given food 3 h after oral administration. Lefucoxib was administered by gastric gavage at the dose of 75 mg kg^{-1} as a 0.5% carboxymethyl-cellulose suspension. Blood samples (about 0.4 ml) were collected in heparinized polythene tubes before administration and post-dose at 0.3, 1.0, 1.7, 2.3, 3.0, 4.0, 6.0, 8.0, 10.0, 12.0, 18.0, 24.0, 36.0, and 48.0 h. The plasma was separated out by centrifugation at 6000 rpm for 10 min and was stored at −20 °C until analysis.

Excretion was studied in another five Wistar rats (male, weighing 250 ± 10 g), raised in cages separately. Rats were housed with free access to food and water, except for the final 12 h before a single oral administration of 75 mg kg^{-1} of lefu-

coxib (access to water was ad libitum during the experiment). Feces and urine were collected after administration in different periods (0–12, 12–24, 24–36, 36–48 h). The amount of feces and urine collected over each period was recorded, respectively, and then feces and urine was stored at −20 °C until analysis.

2.7. Data analysis and pharmacokinetic calculations

Concentrations of lefecoxib in all biological matrices were calculated using the Excel program (Microsoft Corp., USA). Pharmacokinetic parameters were calculated using non-compartmental methods. The area under the plasma concentration versus time curve up to the last quantifiable time point, $AUC_{0–36\,h}$, was calculated by the linear trapezoidal rule. The maximum observed plasma concentration (C_{max}) and the time of maximum observed plasma concentration (T_{max}) were obtained directly from the concentration–time curve. $T_{1/2\beta}$ was calculated as $0.693/K_e$ (K_e was determined by linear regression of the terminal log-linear phase of the concentration versus time curve points). The mean residence time (MRT) was cal-

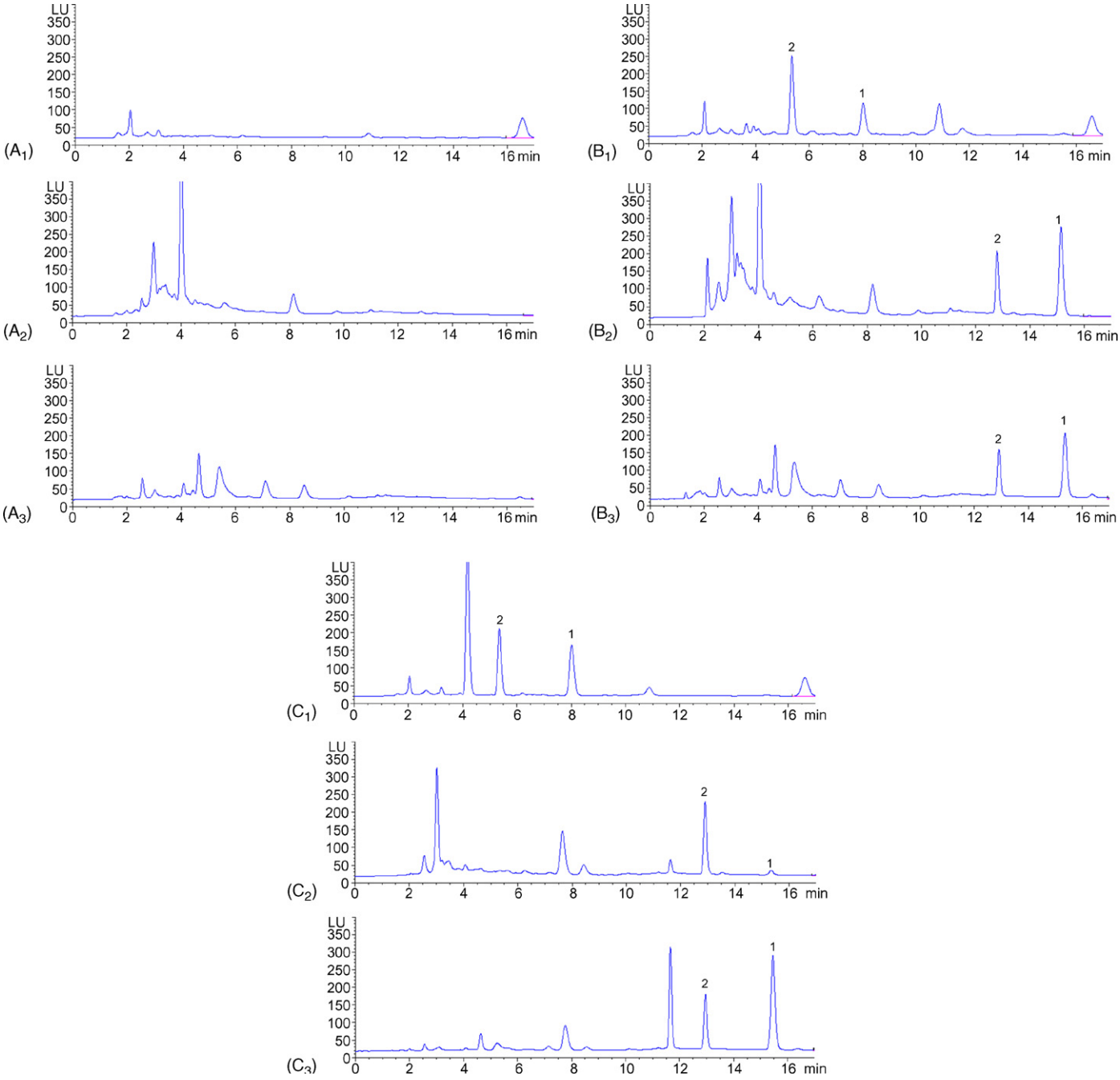

Fig. 2. Chromatograms of lefucoxib and I.S. in rat plasma, urine, and fecal homogenate: (A_1) blank rat plasma; (A_2) blank urine; (A_3) blank fecal homogenate; (B_1) blank plasma spiked with lefucoxib and I.S.; (B_2) blank urine spiked with lefucoxib and I.S.; (B_3) blank fecal homogenate spiked with lefucoxib and I.S.; (C_1) a rat plasma sample after oral administration; (C_2) a urine sample after oral administration; (C_3) a fecal homogenate sample after oral administration; (1, lefucoxib; 2, I.S.).

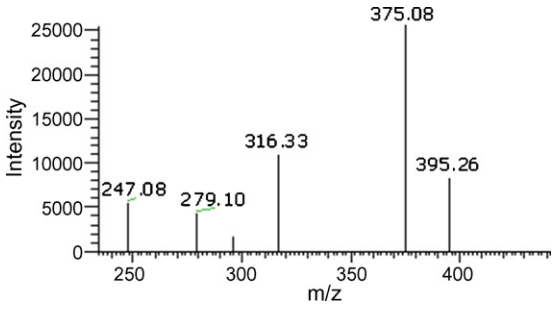

Fig. 3. Mass spectra of lefucoxib after the separation.

culated from the ratio of total area under the first moment of the drug concentration curve ($AUMC_{0–36\,h}$) to $AUC_{0–36\,h}$. The percentage of lefucoxib eliminated in feces was calculated with the accumulated lefucoxib eliminated in all periods divided by administration amount (lefucoxib eliminated over each period was calculated using the amount of feces multiplied by the concentration of lefucoxib in it).

3. Method validation

3.1. Specificity

The specificity of the method was demonstrated by comparing chromatograms of six independent biological samples (plasma, urine, and fecal homogenate) from blank rats, each as a blank sample and a spiked sample. Fig. 2 indicates no significant interferences at the retention times of lefucoxib and I.S. In plasma samples, the retention times for lefucoxib and I.S. were 8.1 and 5.3 min, respectively. In urine and feces samples, they turned 15.4 and 12.9 min due to the gradient profile.

3.2. Qualitative analysis after the separation

Qualitative analysis of lefucoxib after the separation was carried out by MS/MS spectra with a ThermoFinnigan LCQ Advantage Quadruple Ion Trap Mass Spectrometer with Xcalibur version 1.4 software. Mass 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/MS 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). Single ion monitoring (SIM) modes were used to accomplish the qualitative analysis of lefucoxib.

Positive mass spectra showed the molecular ion ($[M+1]^+$) at *m/z* 395, in agreement with the molecular weight of lefucoxib being 394. Besides, a characteristic MS/MS fragment ion (*m/z* 375, $[395-HF]^+$, Fig. 3) was obtained when *m/z* 395 was selected as a target ion, which was the same as that of lefucoxib standard.

3.3. Linearity and limit of quantification (LOQ)

Biological samples were quantified using the ratio of the peak area of lefucoxib to that of I.S. To evaluate linearity, calibration curves were prepared and assayed in triplicate on three consecutive assays over the range of 5.0–1000.0 ng ml^{-1}. Calibration curves were calculated utilizing the peak-area ratio versus lefucoxib concentration. The response was linear throughout this concentration range and the correlation coefficients (r) were greater than 0.99 for all standard curves using a $1/x^2$ weighted linear regression model. The typical equation was $y = 6.11 \times 10^{-3} + 5.51 \times 10^{-3}x$ ($r = 0.9994$). During routine analysis each analytical run included a set of calibration samples, a set of QC samples and unknowns. The LOQ was 5.0 ng ml^{-1}, determined by analyzing matrix samples spiked with known concentration of analyte that can be quantified with acceptable accuracy and precision (±15%).

3.4. Accuracy and precision

Accuracy and precision were assessed by determination of QC samples at three concentration levels in three different validation assays. The accuracy was expressed by relative recovery and the precision by relative standard deviation (R.S.D.). The method showed good precision and accuracy. Table 1 summarizes the intra- and inter-assay precision and accuracy for lefucoxib from biological matrices QC samples. The intra- and inter-assay precisions were measured to be below 5.5% and 14.2%, respectively, with relative recovery from 97.9% to 108.1%.

Table 1
Summary of precision and accuracy of lefucoxib in rat plasma, urine, and feces ($n = 3$ assays, 5 replicates per assay)

Biological matrix	Added C (ng ml^{-1})	Found C (ng ml^{-1})	Recovery (%)	Intra-assay R.S.D. (%)	Inter-assay R.S.D. (%)
Plasma	10.0	10.8 ± 0.6	108.1	5.5	3.0
	500.0	494.7 ± 22.9	98.9	2.8	10.1
	1000.0	1011.8 ± 64.7	101.2	4.7	12.5
Urine	10.0	10.4 ± 0.5	104.1	4.3	7.4
	500.0	489.5 ± 27.3	97.9	4.4	8.2
	1000.0	1032.7 ± 78.8	103.3	3.3	14.2
Feces	10.0	10.1 ± 0.6	101.0	4.7	9.7
	500.0	517.2 ± 10.3	103.4	1.4	3.2
	1000.0	1065.0 ± 31.5	106.5	2.4	4.2

Table 2
Summary of stability studies of lefucoxib in rat plasma, urine, and feces under various storage conditions ($n = 5$)

Storage conditions	Biological matrix	Added C (ng ml^{-1})	Found C (ng ml^{-1})	R.S.D. (%)	Recovery (%)
Non-processed samples stored for 5 h (20 °C)	Plasma	10.0	10.9 ± 0.7	6.0	109.5
		250.0	265.2 ± 13.9	5.2	106.1
		500.0	507.5 ± 39.3	7.7	101.5
		1000.0	980.8 ± 55.0	5.6	98.1
	Urine	10.0	10.7 ± 0.2	1.7	107.4
		500.0	496.1 ± 31.0	6.2	99.2
		1000.0	1065.4 ± 28.6	2.7	106.5
	Feces	10.0	9.3 ± 0.4	4.0	93.1
		500.0	470.0 ± 19.7	4.2	94.0
		1000.0	929.3 ± 14.0	1.5	92.9
Three freeze/thaw cycles	Plasma	10.0	9.6 ± 0.8	8.1	95.8
		250.0	231.4 ± 7.5	3.3	92.6
		500.0	455.3 ± 21.2	4.7	91.1
		1000.0	1076.9 ± 30.4	2.8	107.7
	Urine	10.0	10.1 ± 0.3	3.0	101.2
		500.0	496.3 ± 10.1	2.0	99.3
		1000.0	1038.0 ± 29.4	2.8	103.8
	Feces	10.0	10.6 ± 0.4	3.5	105.6
		500.0	487.9 ± 11.9	2.4	97.6
		1000.0	953.3 ± 24.0	2.5	95.3
Processed samples stored on the rack of autosampler for 24 h (20 °C)	Plasma	10.0	9.3 ± 0.3	2.9	92.7
		250.0	225.2 ± 6.9	3.1	90.1
		500.0	526.1 ± 33.7	6.4	105.2
		1000.0	977.6 ± 52.3	5.3	97.8
	Urine	10.0	10.8 ± 0.4	4.0	107.8
		500.0	508.5 ± 33.2	6.5	101.7
		1000.0	1044.7 ± 35.6	3.4	104.5
	Feces	10.0	10.6 ± 0.4	3.5	105.6
		500.0	487.9 ± 11.9	2.4	97.6
		1000.0	953.3 ± 24.0	2.5	95.3

3.5. Stability

To 0.19 ml of the blank rat plasma, urine, and fecal homogenate, 0.01 ml of lefucoxib standards were added to yield final concentrations as, 10.0, 250.0, 500.0, and 1000.0 ng ml^{-1} of lefucoxib in plasma samples, and for urine and fecal homogenate that was 10.0, 500.0, and 1000.0 ng ml^{-1}. These QC samples were used to investigate the stability of lefucoxib under the experimental conditions Table 2 shows the stability studies of lefucoxib in rat plasma, urine, and fecal homogenate. The number of replicates employed for each determination was five. Results were expressed for each concentration level as the percentage of the added concentration (C), which was referred to as 100%. Stability was studied for the spiked biological matrices after 5 h at room temperature and for up to three freeze/thaw cycles. Stability of the processed samples stored on the rack of autosampler was also studied after storing for 24 h at room temperature (about 20 °C).

3.6. Absolute recovery

The absolute recoveriy of lefucoxib was determined by spiked samples (plasma, urine, and fecal homogenate) at three concentrations: 10.0, 500.0, and 1000.0 ng ml^{-1}. It was calculated by comparing peak areas from spiked samples to the same amounts of unextracted lefucoxib solutions

Table 3
Absolute recoveries of lefucoxib in rat plasma, urine, and feces with and without the I.S.

Added C (ng ml^{-1})	Plasma without I.S. (%)	Plasma with I.S. (%)	Urine without I.S. (%)	Urine with I.S. (%)	Feces without I.S. (%)	Feces with I.S. (%)
10.0	70.9 ± 4.3	72.9 ± 1.9	73.6 ± 5.3	70.3 ± 3.6	75.6 ± 5.4	74.1 ± 4.4
500.0	63.8 ± 1.5	65.2 ± 1.2	67.5 ± 4.0	69.6 ± 3.2	67.9 ± 2.2	70.5 ± 3.9
1000.0	69.8 ± 2.1	74.3 ± 1.0	70.1 ± 2.7	68.4 ± 4.3	73.7 ± 1.6	70.2 ± 3.4

Mean ± S.D. % ($n = 5$).

Table 4
Pharmacokinetic parameters obtained after administrations of lefucoxib in Wistar rats

T_{max} (h)	C_{max} (ng ml^{-1})	$AUC_{0-36\,h}$ (ng h ml^{-1})	$T_{1/2}$ (h)	MRT (h)	Percentage of lefucoxib eliminated in feces (0–48 h, %)
7.6 ± 1.7	140.4 ± 45.8	1663.0 ± 570	6.4 ± 1.3	10.9 ± 2.3	75.8 ± 15.2

Mean ± S.D. (n = 5).

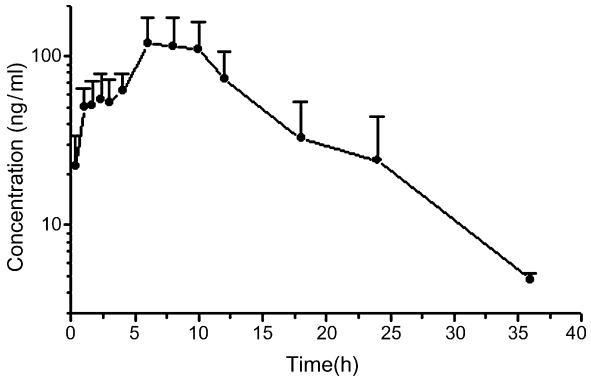

Fig. 4. Mean plasma concentration–time profile of lefucoxib after an oral administration (75 mg kg^{-1}) to five Wistar rats. Each point represented the mean ± S.D.

both with and without I.S., and results are shown in Table 3.

The table shows that there were no significant differences on absolute recovery of lefucoxib among plasma, urine, and fecal homogenate. In addition, the I.S. did not have influence on the absolute recovery of lefucoxib from biological matrices.

4. Application of the method in pharmacokinetic studies

After a single oral administration of lefucoxib (75 mg kg^{-1}) to five Wistar rats, the concentrations of lefucoxib in plasma, urine, and fecal homogenate were determined by the described HPLC fluorescence method. Fig. 4 shows mean plasma concentration–time curves of lefucoxib after administration. The percentage of lefucoxib excreted through feces was about 76% (as Fig. 5 shows) after an oral administration, and trace level of lefucoxib was found in urine (lower than LOQ). Pharmacokinetic parameters are listed in Table 4.

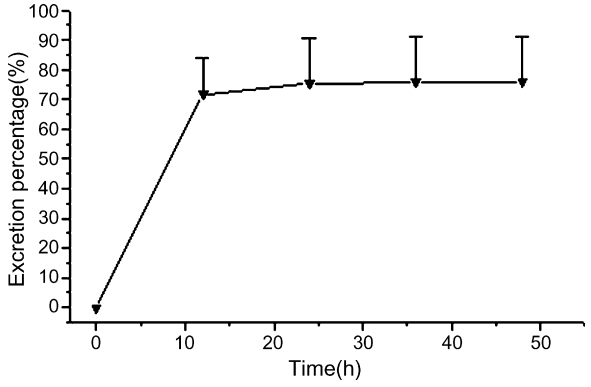

Fig. 5. Mean excretion percentage–time profile of lefucoxib after an oral administration (75 mg kg^{-1}) to five Wistar rats. Each point represented the mean ± S.D.

5. Conclusion and discussion

To develop this assay, many sample preparation methods were tried. It was found that a bad specificity was obtained when the solid-phase extraction was applied. Much interference made it impossible to detect lefucoxib specifically although a little better absolute recovery was obtained. Many kinds of organic solutions were tried to extract lefucoxib and I.S. from biological matrices, such as methanol, acetonitrile, and acetic ether. Again, a bad specificity was obtained.

Before fluorescence detection was chosen to determinate lefucoxib and I.S, the UV detection had been tried. Compared with fluorescence detection it supplied bad both sensitivity and specificity.

In the assay, blood samples were eluted from the analytical column by an isocratic profile (methanol–water (80:20, v/v)). However, a gradient profile was carried out to elute lefucoxib and I.S. in urine and fecal homogenate samples. That was because much more endogenous components were extracted from urine and feces, and they resulted in many interfering peaks at the same retention times of analytes without the gradient elution.

In conclusion, the described HPLC fluorescence method was a sensitive, accurate, and specific assay for the determination of lefucoxib in rat plasma, urine, and feces. The small amount of biological matrices required (0.2 ml per determination) made this method suitable for routine analysis in preclinical pharmacokinetic studies, and the method might be helpful in clinical pharmacokinetic studies. It can also be used as a reference for therapeutic drug monitoring of lefucoxib and other COX-2 inhibitors.

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