Journal of Pharmaceutical and Biomedical Analysis 50 (2009) 41–45

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Validated LC–MS/MS assay for the determination of felbinac: Application to a preclinical pharmacokinetics study of felbinac trometamol injection in rat

Chao Zhang [a], Lu Wang [a], Wei Yang [b], Xisha Wang [b], J. Paul Fawcett [c], Yantong Sun [a,d], Jingkai Gu [a,*]

[a] Research Center for Drug Metabolism, College of Life Science, Jilin University, Changchun 130021, PR China
[b] Guangdong Pharmaceutical University, Guangzhou 510006, PR China
[c] School of Pharmacy, University of Otago, P.O. Box 913, Dunedin, New Zealand
[d] School of Pharmaceutical Sciences, Jilin University, Changchun 130021, PR China

ARTICLE INFO

Article history:
Received 21 February 2009
Received in revised form 17 March 2009
Accepted 18 March 2009
Available online 26 March 2009

Keywords:
Felbinac
Felbinac trometamol
LC–MS/MS
Preclinical study
Pharmacokinetics
Rat

ABSTRACT

A rapid and sensitive analytical method based on high-performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) has been developed for the determination of felbinac in rat plasma, bile, urine, feces and tissue. Sample preparation involved liquid–liquid extraction with ethyl ether–dichloromethane (60:40, v/v). Chromatography of felbinac and the internal standard probenecid was performed within 2 min on a Venusil MP C_{18} column (100 mm × 4.6 mm i.d., 5 μm) with a mobile phase consisting of acetonitrile–5 mM ammonium acetate containing 0.1% formic acid (pH 3.0) (80:20, v/v) at a flow rate of 1.2 ml/min. Detection by electrospray negative ionization mass spectrometry and multiple-reaction monitoring of the transitions of felbinac at *m/z* 211.1 → 167.0 and of probenecid at *m/z* 283.9 → 239.9 was linear over the concentration range 5–5000 ng/ml with a lower limit of quantitation of 5 ng/ml using a sample volume of only 50 μl. Intra- and inter-day precisions (as relative standard deviation, R.S.D.) were ≤7.3% and ≤6.4%, respectively, and accuracy (as relative error, R.E.) was in the range −2.1 to 7.4%. Recoveries and matrix effects were satisfactory in all the biological matrices examined. The method was applied to a preclinical pharmacokinetic study in rat involving a single intravenous injection of felbinac trometamol.
© 2009 Elsevier B.V. All rights reserved.

1. Introduction

In the clinical field, nonsteroidal anti-inflammatory drugs (NSAIDs) remain the mainstay of therapy for osteoarthritis, rheumatoid arthritis and acute pain. The desirable effects of NSAIDs result from the inhibition of cyclooxygenase-2 (COX-2) [1,2] but non-selective NSAIDs are associated with undesirable gastrointestinal and renal toxicity due to concomitant inhibition of COX-1 [3–5]. NSAIDs are commonly used orally to treat rheumatoid arthritis, topically to treat muscle inflammation and intravenously for post-operative pain.

Felbinac (2-(4-phenylphenyl)acetic acid) (Fig. 1A) is a non-selective arylpropionic acid type NSAID traditionally used topically [6–8]. Recently an intravenous (i.v.) formulation has been developed based on the highly water soluble trishydroxymethylaminomethane salt known as felbinac trometamol (Fig. 1B). Initial investigations (unpublished data) indicate this new salt is an effective antipyretic and analgesic which can be formulated as an intravenous injection without the need for macromolecular adjuvants such as lecithin used in the commercially available felbinac ethyl injection (Daitac, Lederle, Japan). In order to carry out preclinical pharmacokinetic studies of this salt, we have developed a rapid and sensitive analytical method for felbinac.

Several methods based on high performance liquid chromatography (HPLC) with UV or fluorescence detection [9–11] for the simultaneous determination of felbinac and other compounds in human plasma or urine have been previously reported. However, they possess a number of disadvantages such as low sensitivity (≥50 ng/ml) leading to the need for a large sample volume (≥1 ml), long retention time (≥7 min) and/or poor selectivity when applied to complex matrices such as bile, feces and tissues. A published gas chromatography–mass spectrometry assay with precolumn derivatization [12] for quantitation of felbinac in human plasma and synovial fluid involved tedious and time-consuming sample preparation and a long analytical run time. In order to facilitate preclinical studies in rat where large sample volumes are not available, we developed an assay based on liquid chromatography–tandem mass spectrometry (LC–MS/MS), a method requiring only 50 μl of sample and employing simple sample treatment but capable of high specificity and sensitivity as well as high sample throughput. The method was fully validated for biological fluids, feces, subcutaneous fat and skeletal muscle and applied to a preclinical pharmacokinetic study of felbinac trometamol intravenous injection in rat.

* Corresponding author. Tel.: +86 431 85619955.
E-mail address: gujk@mail.jlu.edu.cn (J. Gu).

0731-7085/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2009.03.019

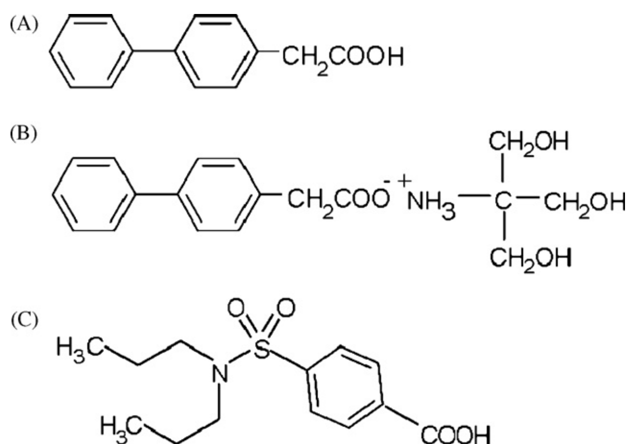

Fig. 1. Structures of (A) felbinac, (B) felbinac trometamol and (C) probenecid.

2. Experimental

2.1. Materials

Felbinac reference standard (purity > 99.5% by HPLC) and ampoules of felbinac trometamol injection (22.5 mg/ml) were obtained from Guangdong Zhongke Drug Research & Development Co. Ltd (Guangzhou, PR China). Probenecid (Fig. 1C) for use as internal standard (I.S.) was kindly supplied by Changchun Gallop Medicine Co. Ltd. (Changchun, PR China). HPLC grade methanol and acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ, USA). All other chemicals were of analytical grade and used without further purification. Distilled water prepared from demineralized water was used. Blank samples (drug free) of rat plasma, urine, feces, bile, skeletal muscle and subcutaneous fat were obtained from the Pharmaceutical Industrial Research Institute (Guangzhou, PR China).

2.2. LC–MS/MS

Liquid chromatography was performed on an Agilent 1100 system (Agilent, Waldbronn, Germany) consisting of a G1311A quaternary pump, a G1379A vacuum degasser, a G1316A thermostatted column oven maintained at 40 °C and a G1313A autosampler maintained at 18 °C. Separation was achieved on a Venusil MP C_{18} column (100 mm × 4.6 mm i.d., 5 μm) preceded by a SecurityGuard C_{18} guard column (4 mm × 3.0 mm i.d., Phenomenex, Torrance, CA, USA). Isocratic elution employed a mobile phase of acetonitrile-5 mM ammonium acetate containing 0.1% formic acid (pH 3.0) (80:20, v/v) at a flow rate of 1.2 ml/min. A split of the eluent was included to allow only 0.6 ml/min to enter the mass spectrometer. The total run time of the assay was 2.0 min.

Mass spectrometric detection was carried out on an API 4000 triple quadrupole instrument (Applied Biosystems, Concord, Ontario, Canada) equipped with a TurboIonSpray (ESI) source operated in the negative ion mode. MS parameters for felbinac and I.S. at unit resolution were optimized by direct infusion of standard solutions (100 ng/ml) into the mass spectrometer at a flow rate of 20 μl/min. Multiple-reaction monitoring (MRM) used the transitions of the deprotonated molecules at m/z 211.1 → 167.0 for felbinac and m/z 283.9 → 239.9 for probenecid with a scan time of 200 ms per transition. Optimal values of MS parameters were as follows: nebulizer, heater and curtain gas (all nitrogen) flow rates 40, 30 and 15 arbitrary units, respectively; ionspray voltage −3500 V; source temperature 400 °C; collision gas, 5 units; collision energies and declustering potentials (respectively) −10 eV and −30 V for felbinac and −20 eV and −50 V for probenecid. Data acquisition and integration were controlled by Analyst 1.3.2 software (Applied Biosystems).

2.3. Preparation of calibration standards and quality control samples

Stock solutions of felbinac (1 mg/ml) and I.S. (0.5 mg/ml) were prepared in methanol. A series of felbinac standard solutions with concentrations of 0.25, 0.75, 2.5, 7.5, 25, 75 and 250 μg/ml were prepared by dilution of the stock solution with methanol–water (50:50, v/v). Quality control (QC) solutions were prepared independently at concentrations of 0.75, 7.5 and 75 μg/ml in the same way. A working I.S. solution (0.5 μg/ml) was also prepared in methanol–water (50:50, v/v). All solutions were stored at 4 °C and used within 1 month. Calibration standards of felbinac with concentrations of 5, 15, 50, 150, 500, 1500 and 5000 ng/ml were prepared by mixing 50 μl aliquots of the standard solutions with 2450 μl of blank matrix. QC samples (15, 150, and 1500 ng/ml) were prepared from QC solutions in a similar way. All samples were prepared in bulk, aliquoted, and stored at −20 °C until use.

2.4. Sample preparation

A 50 μl aliquot of rat plasma was mixed with 50 μl I.S. solution and 50 μl 1 M HCl before being extracted with 3 ml ethyl ether–dichloromethane (60:40, v/v) by shaking for 10 min. Following centrifugation at 3500 × g for 5 min, the organic layer was transferred to another tube and placed in an evaporator (Zymark, Hopkinton, MA, USA) at 40 °C under a gentle stream of nitrogen. The residue was reconstituted in 100 μl mobile phase and vortex-mixed for 30 s after which 20 μl was injected into the LC–MS/MS system. Plasma samples with concentrations greater than the maximum calibration standard were determined after dilution with blank plasma. Tissue samples and dried samples of feces were accurately weighed, mixed with 4 ml/g methanol and homogenized. Tissue and feces homogenates, bile and urine samples were then prepared as described for plasma samples.

2.5. Assay validation

Validation was performed for samples of plasma, bile, feces, urine, skeletal muscle and subcutaneous fat according to FDA Guidance for Industry, Bioanalytical Method Validation [13]. Specificity was assessed by analysis of six different samples of blank matrix with and without spiking with I.S. and felbinac. Linearity was assessed by weighted ($1/x^2$) least squares linear regression of calibration curves generated in triplicate on three consecutive days using analyte: I.S. peak area ratios. Intra- and inter-day precision (R.S.D.) and accuracy (R.E.) were determined by analysis of six replicate QC samples on three different days and inverse prediction of the concentrations from the calibration curve. The acceptance criteria for each back calculated concentration were precision <15% and accuracy <15% of the nominal value. The LLOQ was defined as the lowest concentration that could be determined with acceptable precision (±20%) and accuracy (±20%). Recoveries were estimated by comparing the peak areas of felbinac in six replicates of QC samples with those of post-extraction blank matrix extracts of the corresponding matrices spiked at the corresponding concentrations. Matrix effects of felbinac for all matrices were evaluated by comparing the peak areas of post-extraction blank plasma spiked at concentrations of QC samples with the areas obtained by direct injection of corresponding standard solutions. Recovery and matrix effect of probenecid were assessed similarly as those of felbinac using six replicates of matrices spiked with I.S. (0.5 μg/ml). Stability of felbinac in the six matrices was established by analysis of three replicates of QC samples under the following conditions:

Long-term stability at −20 °C for three months; short-term stability at 25 °C for 12 h; in processed samples in autosampler vials for 12 h; and after three freeze/thaw cycles (−20 to 25 °C). The effect of dilution was evaluated for the analysis of plasma samples containing felbinac at concentrations higher than the upper limit of the standard curve by analysing six replicates of rat plasma spiked with felbinac at 50 μg/ml and diluting with blank rat plasma to three concentration levels (15, 150, 1500 ng/ml).

2.6. Pharmacokinetic study

The assay was applied to a preclinical pharmacokinetic study of felbinac administered as felbinac trometamol injection. Sprague–Dawley rats (Pharmaceutical Industrial Research Institute, Guangzhou, PR China) ($n = 26$, weight 250 ± 10 g) were randomly divided into three groups containing equal numbers of males and females and maintained in individual metabolic cages at 25 °C with

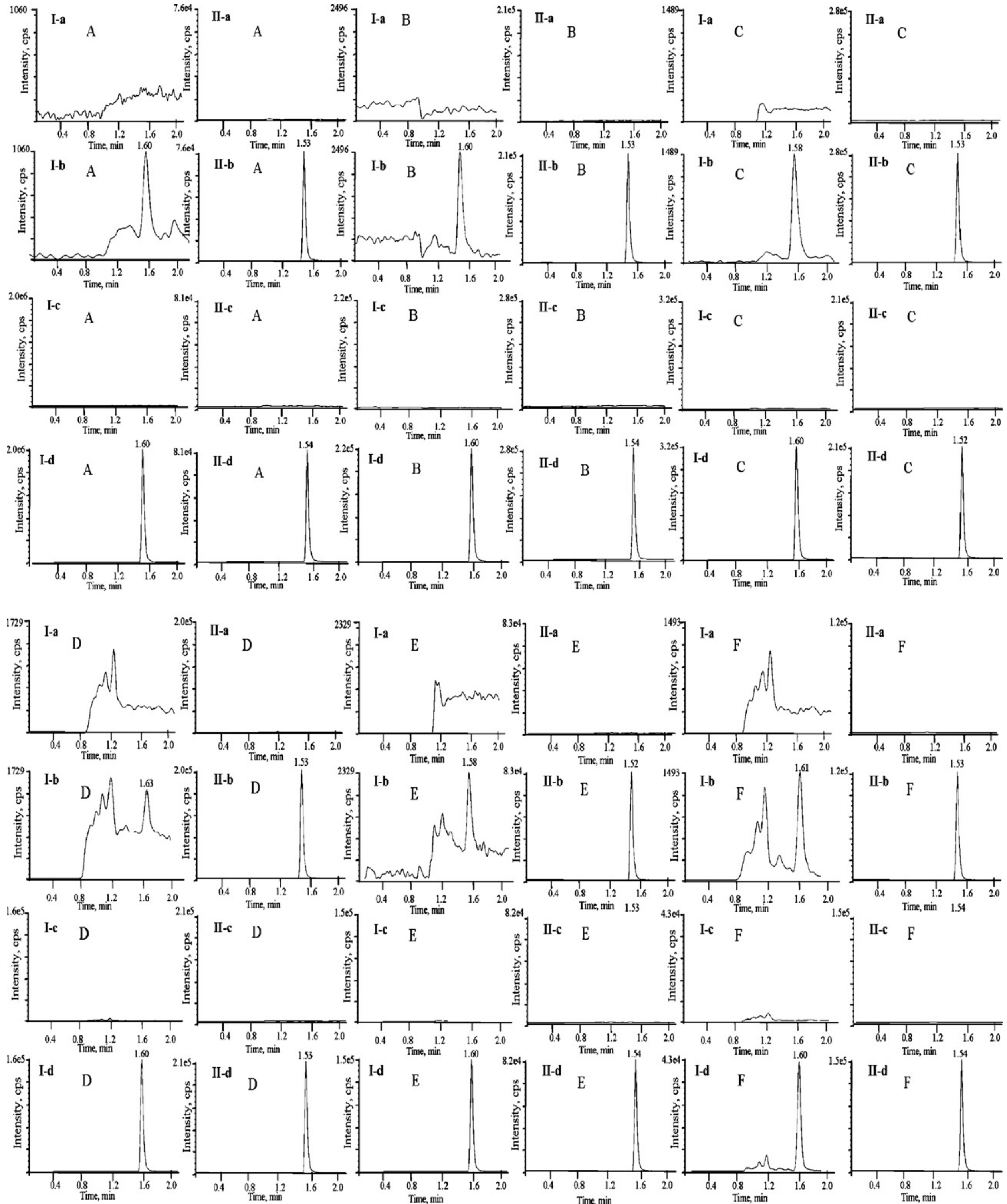

Fig. 2. Typical MRM chromatograms of (I) felbinac and (II) I.S. in six rat matrices. (A) Plasma, (B) skeletal muscle, (C) subcutaneous fat, (D) feces, (E) bile, (F) urine. (a) Blank chromatograms with the same sensitivity as spiked samples; (b) samples spiked with felbinac 5 ng/ml and I.S. 0.5 μg/ml; (c) blank chromatograms with the same sensitivity as real samples (d) real samples obtained after a single i.v. dose (8.4 mg/kg) of felbinac trometamol.

Table 1
Accuracy and precision for the analysis of felbinac in plasma (Data are based on assay of six replicates of QC samples and a sample at the LLOQ on three consecutive days).

	Concentration (ng/ml)		Precision (R.S.D. %)		Accuracy (R.E. %)
	Nominal	Found mean ± SD	Intra-day	Inter-day	
Plasma	5 (LLOQ)	4.88 ± 0.22	1.8	4.9	−2.4
	15	14.7 ± 0.7	3.9	5.1	−1.8
	150	147 ± 4	4.6	2.4	−2.1
	1500	1560 ± 37	4.5	1.9	3.8

a 12 h light/dark cycle. Of the 26 rats, 6 were used to collect plasma, urine and feces samples, 16 to collect tissue samples and 4 to collect bile samples after bile duct cannulation under general anesthesia. After a 12 h fast, rats were administered a single i.v. dose (tail vein) of felbinac trometamol injection (8.4 mg/kg felbinac trometamol) as a solution (4.2 mg/ml) prepared by dilution of the original injection formulation with physiological saline. Blood samples were collected into heparinized test tubes before the dose and at 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24 and 36 h after dosing. Plasma was separated by centrifugation at 3500 × *g* for 10 min and stored at −20 °C until analysis. Tissue samples and cumulative urine, feces, bile samples were collected at various times up to 48 h after the dose. Feces samples were dried and then all samples were stored at −20 °C until analysis. The protocol was approved by the Ethics Committee of the Pharmaceutical Industrial Research Institute, Guangzhou, PR China.

3. Results and discussion

3.1. Assay development

Probenecid was selected as the I.S. due to its similar chromatographic behavior and extraction efficiency to those of felbinac. In Q1 full scan MS, felbinac and probenecid predominantly formed deprotonated molecular $[M-H]^-$ ions at *m*/*z* 211.1 and *m*/*z* 283.9, respectively. In full scan product ion MS, felbinac produced only one fragment at *m*/*z* 167.0 resulting from the loss of carbon dioxide. The product ion mass spectrum of probenecid (*m*/*z* 283.9) included peaks at *m*/*z* 239.9 and 139.9, where the former, produced by loss of carbon dioxide, was the most intense. The transitions *m*/*z* 211.1 → 167.0 and *m*/*z* 283.9 → 239.9 were therefore selected for MRM of felbinac and probenecid, respectively.

A number of commercially available reversed phase HPLC columns (Zorbax extend C_{18}, Hypersil C_{18} and Venusil MP C_{18}) were evaluated and Venusil MP C_{18} (100 mm × 4.6 mm i.d., 5 μm) chosen on the basis of superior chromatography at a flow rate of 1.2 ml/min. In terms of the mobile phase composition, acetonitrile gave a better response than methanol and 5 mM ammonium acetate containing 0.1% formic acid (pH 3.0) provided good resolution with excellent peak shapes and satisfactory mass spectrometric responses.

In investigating sample preparation, simple protein precipitation provided acceptable recovery but was associated with severe matrix effects especially in the analysis of bile, feces and tissue samples. Solvent extraction of acidified samples was eventually adopted because it produced clean extracts while simultaneously concentrating the drug. Of several organic solvents tested (ethyl ether, dichloromethane, ethyl acetate, hexane and combinations thereof), ethyl ether–dichloromethane (60:40, v/v) gave good recoveries with acceptable matrix effects.

3.2. Assay validation

Fig. 2 shows representative MRM chromatograms of felbinac and I.S. in the six rat matrices. The data show there was no significant interference at the retention times of felbinac (1.60 min) or I.S. (1.52 min). In addition, chromatograms allowed to run out to 10 min showed no late-eluting interfering peaks.

Calibration curves were linear over the concentration range 5–5000 ng/ml in all matrices with correlation coefficients (r) > 0.995 and LLOQ of 5 ng/ml. Analysis of a plasma sample spiked at 50 μg/ml and diluted to concentrations of 15, 150 and 1500 ng/ml gave values of 97.9–103%, 98.3–104% and 97.2–103% respectively of the nominal concentration.

Table 1 summarizes accuracy and precision for the analysis of felbinac in rat plasma based on analysis of QC samples. Intra- and inter-day precisions (R.S.D.) in other matrices were ≤7.3% and ≤6.4%, respectively with accuracy (R.E.) in the range −2.1 to 7.4%.

Corresponding values of precision at the LLOQ were ≤ 5.9% and ≤ 4.9% respectively, with accuracy of −0.9 to 2.8%. The extraction recoveries of felbinac at 15, 150 and 1500 ng/ml in the six matrices were all in the range 72.3–104.9%; the extraction recoveries of I.S. at 0.5 μg/ml were in the range 78.1–97.2%.

Studies of matrix effects of felbinac at concentrations of 15, 150 and 1500 ng/ml gave concentrations within ±10% of nominal values for all matrices except feces where values were somewhat low (77.1–78.6%). The I.S. at 0.5 μg/ml gave concentrations in the range 89.0–105.3% of the nominal value in the six matrices.

Table 2 presents the results of the stability study in rat plasma with decomposition <15% under all the conditions examined. Similar results were obtained in the other matrices.

3.3. Pharmacokinetic study

The assay was applied to the determination of felbinac in a preclinical pharmacokinetic study of felbinac administered as felbinac trometamol (i.v.) injection. The mean concentration–time profile for felbinac in plasma is shown in Fig. 3. The plasma concentration immediately after i.v. administration (C_0) was 25.7 ± 4.3 μg/ml, followed by elimination with a half-life ($t_{1/2}$) of 3.22 ± 1.66 h. The area under the plasma concentration–time curve (AUC_{0-t}) was 66.7 ± 37.9 μg h/ml. Calculated pharmacokinetic parameters showed that the steady-state volume of distribution (V_{ss}) of felbinac was 0.48 ± 0.07 l/kg, and the clearance 2.57 ± 1.04 ml/min/kg. Felbinac was rapidly absorbed into tissue with concentrations of

Table 2
Stability of felbinac in rat plasma and processed QC samples (Data are mean ± SD, n = 3).

Nominal concentration (ng/ml)	Long-term (−20 °C for 3 months)		Short-term (25 °C for 12 h)		Freeze/thaw stability (3 cycles−20 °C to 25 °C)		Processed QC samples (18 °C for 12 h)	
	Mean ± SD	R.E.(%)	Mean ± SD	R.E.(%)	Mean ± SD	R.E.(%)	Mean ± SD	R.E.(%)
15	14.73 ± 0.40	−1.8	15.47 ± 0.47	3.1	15.33 ± 0.93	2.2	15.53 ± 0.50	3.6
150	149.3 ± 2.1	−0.4	151.6 ± 2.1	1.1	152.6 ± 1.7	1.7	153.6 ± 3.1	2.4
1500	1513 ± 15	0.9	1560 ± 17	4.0	1557 ± 21	3.8	1563 ± 21	4.2

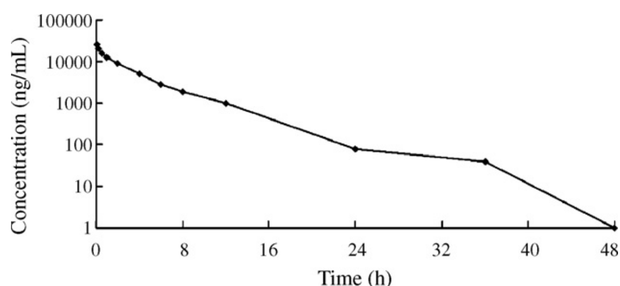

Fig. 3. Mean plasma concentration–time curve of felbinac after a single intravenous dose (8.4 mg/kg) of felbinac trometamol.

494 and 374 ng/g appearing in subcutaneous fat and skeletal muscle respectively within 5 min of the i.v. dose. Concentrations in urine, feces and bile indicate that <1% of the dose is excreted unchanged suggesting felbinac undergoes extensive hepatic metabolism in rat following i.v. administration. However, the presence of parent drug in the bile and subsequently in the feces suggests some transporter mediated efflux occurs at the bile canaliculus.

4. Conclusions

This paper reports the development and validation of a versatile, rapid and sensitive LC–MS/MS assay for the determination of felbinac in rat plasma and other biological matrices. The method was successfully applied to a preclinical pharmacokinetic study of felbinac administered as an intravenous injection of felbinac trometamol, a new formulation of this established NSAID. The assay involves simple sample preparation and has a wide linear concentration range (5–5000 ng/ml), short run time (2 min) and high sensitivity using a small sample volume (50 μl).

Acknowledgements

This work was supported by the Innovation Fund for Small Technology-based Firms and the Ministry of Science and Technology, PR China (item code: 08C26214401254).

References

[1] W.L. Xie, J.G. Chipman, D.L. Robertson, R.L. Erikson, D.L. Simmons, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 2692–2696.
[2] T. Hla, K. Neilson, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 7384–7388.
[3] G. Singh, D.R. Ramey, D. Morfeld, H. Shi, H.T. Hatoum, J.F. Fries, Arch. Intern. Med. 156 (1996) 1530–1536.
[4] S. Hernández-Díaz, L.A. García-Rodríguez, Am. J. Med. 110 (Suppl. 3A) (2001) 20S–27S.
[5] D.H. Solomon, J.H. Gurwitz, Am. J. Med. 102 (1997) 208–215.
[6] E.L. Tolman, R. Partridge, Prostaglandins 9 (1975) 349–359.
[7] T. Shiba, Y. Shikata, N. Takagawa, Jpn. Pharmacol. Ther. 20 (1992) 81–94.
[8] N. Shinkai, K. Korenaga, H. Takizawa, H. Mizu, H. Yamauchi, J. Pharm. Pharmacol. 60 (2008) 71–76.
[9] G. Carlucci, P. Mazzeo, G. Palumbo, J. Chromatogr. B 682 (1996) 315–319.
[10] M. Siluveru, J.T. Stewart, J. Chromatogr. B 682 (1996) 89–94.
[11] T. Hirai, S. Matsumoto, I. Kishi, J. Chromatogr. B 692 (1997) 375–388.
[12] M. Dawson, C.M. Mcgee, P.M. Brooks, J.H. Vine, E. Lacey, T.R. Watson, J. Chromatogr. 420 (1987) 129–134.
[13] US Food and Drug Administration, Guidance for Industry, Bioanalytical Method Validation, Centre for Drug Evaluation and Research, CDER, Rockville, 2001.