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Cloud point extraction-HPLC method for determination and pharmacokinetic study of flurbiprofen in rat plasma after oral and transdermal administration

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ABSTRACT

A method based on cloud-point extraction (CPE) was developed for the determination of flurbiprofen (FP) in rat plasma after oral and transdermal administration by high-performance liquid chromatography coupled with UV detection (HPLC–UV). The non-ionic surfactant Genapol X-080 was chosen as the extract solvent. Variables parameter affecting the CPE efficiency were evaluated and optimized. Chromatography separation was performed on a Diamond C_{18} column (4.6 mm i.d. × 250 mm, 10 μm particle size) by isocratic elution with UV detection at 254 nm. The assay was linear over the range of 0.2–50 and 0.1–10 μg/ml for oral and transdermal administration, respectively, and the lower limit of quantification (LLOQ) was 0.1 μg/ml. The extraction recoveries were more than 84.5%, the accuracies were within ±3.8%, and the intra- and inter-day precisions were less than 10.1% in all cases. After strict validation, the method indicated good performance in terms of reproducibility, specificity, linearity, precision and accuracy, and it was successfully applied to the pharmacokinetic study of flurbiprofen in rats after oral and transdermal administration.

1. Introduction

Flurbiprofen (FP), 2-(2-fluoro-4-biphenyl) propionic acid, is a chiral non-steroidal anti-inflammatory drug (NSAID) of the 2-arylpropionic acid class, which has good analgetic, anti-inflammatory and antipyretic effects [1]. It demonstrates comparable efficacy to other NSAIDs, e.g. aspirin, indomethacin, ibuprofen, naproxen, and diclofenac, which is effectively used in the treatment of rheumatoid arthritis [2]. FP is also indicated for the management of vernal keratoconjunctivitis [3], post-operative ocular inflammation [4], herpetic stromal keratitis [5], excimer laser photorefractive keratectomy [6] and ocular gingivitis [7]. Recent reports suggest potential topical and systemic use of FP in inhibition of colon tumor [8], pain management after foot surgery [9] and peridontal surgery [10]. With its ever increasing use, methods for its quantification in biological fluids have attracted the attention of many investigators. Various methods have been reported for the determination of FP either alone or together with their metabolites in plasma/serum [11–17] or in urine [18,19]. But the sample preparation involved in all these experiments was tedious traditional liquid–liquid extraction, which has numerous drawbacks such as the use of large amounts of toxic and flammable organic solvents, the analyte lose during the evaporation of solvents and the unavoidable adsorption of non-polar analytes to glass surfaces. As an alternative to solvent extraction methods, cloud-point extraction (CPE) which overcomes most of the difficulties in the previous methods, is being used by analytical chemists because of its efficiency, cost effectiveness and environment friendliness [20].

CPE is based on the fact that aqueous solutions of several non-ionic surfactants present clouding behavior when the experimental temperature is appropriately altered. The critical temperature, called "cloud point" depends on the amphiphile nature and concentration of surfactant. When the temperature rise above the cloud-point, the phase separation produce a surfactant-rich phase enriching the analyte (at a very small volume) and supernatant aqueous phase (bulk amount) that withholds a concentration of surfactant close to the critical micellar concentration (CMC) [21]. The hydrophobic analytes of the solution are extracted into the surfactant-rich phase. Compared to the initial solution volume, the surfactant-rich phase volume is very small, thus a high enrichment factor can be obtained [22–26]. After clean-up or dilution, the analyte/surfactant-rich phase permits analysis and quantification of the analyte by techniques such as high performance liquid chromatography, gas chromatography, capillary electrophoresis [27] and liquid chromatography–mass spectrometry [28]. As an effective enrichment method, CPE has recently been successfully used for the selective extraction of various compounds from biological and environmental media, including estrogens, vitamin A, vitamin E, terazosin hydrochloride, arbidol, kinds of proteins, as well as metal ions [29–35]. Whereas, reports about its applications on how

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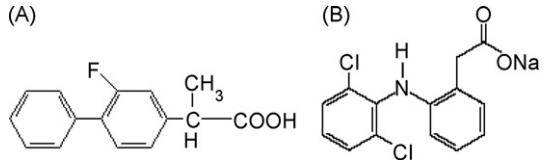

Fig. 1. Chemical structures of flurbiprofen (A) and internal standard (B).

to extract drugs from plasma for clinical and biomedical purpose were so limited.

In order to demonstrate the feasibility of CPE in pharmacokinetic studies of FP, we prepared FP methylcellulose suspension and FP-loaded nanostructured lipid carriers gel (NLC-gel) for oral and transdermal administration, determined FP in rat plasma by CPE preparation using Genapol X-080 as the extraction solvent with HPLC–UV detection, and compared the pharmacokinetic parameters after oral and transdermal administration.

2. Experimental

2.1. Materials and reagents

FP (Fig. 1A) was purchased from Keben Chemical Co. Ltd. (Hangzhou, China). Diclofenac sodium (Fig. 1B), used as internal standard (IS), was obtained from Dongtai Pharm. Co. Ltd. (Henan, China). Non-ionic surfactant oligoethylene glycol monoalkyl ether (Genapol X-080) was purchased from Fluka (New Jersey, USA) and used as received without further purification. HPLC grade methanol and acetonitrile were purchased from Yuwang Chemical Co. Ltd. (Shandong, China). All the other reagents were of analytical grade. Distilled water, prepared with demineralized water, was used throughout the study.

2.2. Instrumentations and chromatographic conditions

Chromatography was preformed using a Shimadzu HPLC system (Kyoto, Japan) consisted of a LC-10AT pump, a Shimadzu UV–VIS detector and a column oven. The separation was carried out on a Diamond C_{18} column (4.6 mm i.d. × 250 mm, 10 μm) (Dikma Technologies). The mobile phase was acetonitrile-0.05 M potassium dihydrogen phosphate solution (60:40, v/v) adjusted to pH 3.5 with phosphoric acid. The flow rate was 1.0 ml/min. The UV detector wavelength was set at 254 nm, and the column temperature was kept at 30 °C.

A thermostatted shaking water bath (HH-2, Guohua Medical Instrument Company, China) was used to implement cloud point extraction. Centrifugation (TDL-16C, Shanghai Anting Medical Instrumental Factory, China) with calibrated centrifugal tubes was applied to accelerate the phase separation process. A vortex mixer (CAY-1, Beijing Chang'an Instrumental Factory, China) was utilized to blend the solution adequately.

2.3. Preparation of standard and quality control samples

A stock solution of FP (200 μg/ml) was prepared in methanol and a series of standard solutions were obtained by further dilution of the stock solution with methanol. A stock solution of diclofenac sodium (internal standard) was prepared in methanol at 80 μg/ml. All solutions were stored at 4 °C.

Calibration standards in plasma were prepared daily by spiking 50 μl of the standard solutions into 200 μl blank rat plasma. The concentrations of FP standard plasma samples were 0.2, 0.5, 1.0, 2.5, 5.0, 10, 20 and 50 μg/ml for oral administration and 0.1, 0.25, 0.5, 1.0, 2.5, 5 and 10 μg/ml for transdermal administration, respectively.

The quality control (QC) samples used in the validation and during the pharmacokinetic studies were prepared in the same way as the calibration standard at appropriate concentrations.

2.4. Sample preparation by CPE procedure

For the extraction and preconcentration of FP, 200 μl of rat plasma sample, 50 μl of methanol and 50 μl of internal standard solution (80 μg/ml) were added to a 1.5 ml tapered centrifuge tube. To this, 1 ml aqueous solution of Genapol X-080 at the concentration of 5% (w/v) and 200 μl HCl (2 mol/l) were added. The tube was vortex-mixed adequately for 5 min and then incubated in a thermostatted shaking water bath at 50 °C for 20 min. The phase separation was accelerated by centrifugation at 3500 rpm for 5 min. The water phase was then removed and the surfactant-rich phase stuck to the wall of the tube was obtained. Coextractants such as hydrophobic proteins and most of the surfactant were removed from the surfactant-rich phase by precipitation with 200 μl of acetonitrile. After centrifugation at 15,000 rpm for 5 min, 20 μl of the supernatant was injected into the HPLC system for analysis.

2.5. Method validation

In order to determine the linearity of the method, calibration standards were prepared in triplicate and analyzed in three separate analytical runs. Daily calibration curves were constructed using the peak-area ratios of FP and the internal standard versus the FP concentrations with least-squares linear regression analysis. The unknown sample concentrations were calculated from the linear regression equation of the peak area ratio against concentrations of the calibration curve.

To determine precision and accuracy, QC samples were prepared at three concentration levels (oral: 0.5, 10 and 40 μg/ml, transdermal: 0.25, 2.5 and 8 μg/ml). Six replicates were analyzed in each of three analytical runs. The precision was evaluated by the relative standard deviation (R.S.D.); the accuracy was determined by calculating the percentage deviation of the observed concentrations from the nominal concentrations and expressed as relative error (R.E.). The precision and accuracy were required to be within ±20% for the lower limit of quantification (LLOQ) and within ±15% for other concentrations. Recovery of the extraction procedure was evaluated at low, medium and high concentrations for FP, and at 80 μg/ml for the IS. It was determined by comparing the mean peak areas ($n = 6$ at each concentration) obtained from plasma samples spiked before extraction to those from plasma samples spiked after extraction.

To evaluate the stability of FP in rat plasma, spiked QC samples of three different concentrations were subjected to three freeze–thaw (−20–25 °C) cycles or were stored at room temperature for 4 h before sample processing. The post-preparative stability storage at 4 °C for 24 h and the long-term stability storage at −20 °C for 14 days were also studied. Stability was assessed by comparing the mean concentration of the stored QC samples with the mean concentration of those prepared freshly. Samples were to be regarded as stable if bias of them were within ±15% of the actual value.

2.6. Application of the method and pharmacokinetic study

Male Wister rats (250–280 g) purchased from the Experimental Animal Center of Shenyang Pharmaceutical University, were kept in an environmental controlled breeding room for 3 days before starting the experiment. All procedures involving animals were in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of People's Republic of China.

Before drug administration, the rats were fasted overnight with free access to water. FP methylcellulose suspension and NLC-gel were prepared and administrated orally (10 mg/kg) and transdermally (10 mg/kg), respectively. Blood samples (0.5 ml) were collected from the ocular vein into heparinized tube before (0 h) and 0.5, 1, 2, 4, 6, 8, 12, 24 and 48 h after dosing, and then immediately centrifuged at 3500 rpm for 10 min. The plasma samples were transferred into new tubes and stored frozen at −20 °C until analysis. Because of a large volume of blood sample was required, 24 rats were divided into four groups of six animals each. Blood was collected from each group at five time points.

The plasma concentrations of FP at different time points were expressed as mean ± S.D., and the mean concentration-time curves were plotted. All the pharmacokinetic data were calculated using the DAS 2.0 statistical software (Pharmacology Institute of China).

3. Results and discussion

3.1. HPLC–UV conditions

To optimize the chromatographic conditions, the mobile phase system was investigated. Preliminary studies on different mobile phase combinations of phosphate buffer, methanol and acetonitrile were considered. The pH of the mobile phase was also explored. Mobile phases of pH 6.5 or 4.5 yielded tailing peaks. While the pH of the mobile phase was adjusted to 3.5, the peaks became sharp and symmetric. Based on this, acetonitrile and phosphate buffer were selected. The type and molar concentration of phosphate buffer and the ratio between acetonitrile and phosphate buffer were also optimized in order to achieve good resolution and separation of analytes as well as short run time. It was found that a mixture of acetonitrile and 0.05 mol/l potassium dihydrogen phosphate solution (60:40, v/v) adjusted to pH 3.5 with phosphoric acid delivered at a flow rate of 1.0 ml/min could achieve our purpose, and was finally adopted. The maximum absorption wavelength of flurbiprofen and diclofenac sodium was 247 nm and 285 nm, respectively. Taking both of the two compounds into consideration, 254 nm was selected as the detection wavelength in our study [36].

3.2. Optimization of the CPE procedure

3.2.1. Selection of the surfactant

Initially, several surfactants were tried as extraction solvents including Triton X-100, Triton X-114, and Genapol X-080. Nevertheless, compared with Triton X series surfactant, Genapol X-080, which is a polyoxyethylene glycol mono ether-type surfactant that has eight oxyethylene units and tridecyl alkyl moieties, does not absorb above 210 nm and will not interfere with the determination of FP and diclofenac sodium. Thus, Genapol X-080 was chosen as the CPE surfactant in this paper. Its critical micellar concentration (CMC) is 0.05 mmol/l (0.028%, w/v) and cloud point is 42 °C in pure water [37].

3.2.2. Effect of Genapol X-080 concentration

Fig. 2 shows that the extraction recovery of FP from rat plasma samples increased with the increase of surfactant concentration in the concentration range from 1 to 5%, but decreased when the surfactant concentration was in the range of 7.5–15%. Since the phase volume ratio (volume of surfactant-rich phase/volume of aqueous solution, after the extraction step) increases as the amount of surfactant raises, which will cause a dilution of the contents, a compromise between recovery and preconcentration has to be adopted

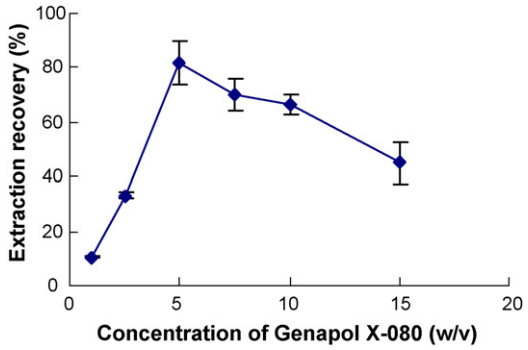

Fig. 2. Effect of concentration of Genapol X-080 on the extraction efficiency ($n = 3$). Experimental condition: 50 °C, 30 min, with addition of 1M HCl.

when using the cloud-point technique [27]. Considering the extraction efficiency and maneuverability, 5% (w/v) Genapol X-080 was selected for further study.

3.2.3. Effect of pH

Solution pH is an important factor during CPE process involving analytes that possess an acidic or basic moiety. For organic molecules, especially for ionizable species, maximum extraction efficiency is achieved at pH values where the uncharged form of the analyte prevails, and therefore, target analyte is favored to be partitioned into the micellar phase. The ionic form of a neutral molecule formed upon deprotonation of a weak acid or protonation of a weak base normally does not interact with and bind the micellar aggregate as strongly as its neutral form does. As a result, a lesser amount of analytes is extracted [38]. In this study, 200 μl of HCl (0.1–2.5 mol/l) was added to the mixture, the effect of which on recovery can be evaluated from Fig. 3. The highest extraction was achieved when the concentration of HCl was 2 mol/l.

3.2.4. Effect of equilibration time and temperature

The effect of incubation time on the extraction efficiency was studied by varying the incubation time from 5 to 60 min. The results indicated that the extraction recovery of FP increased with the increase of extraction time. As can be seen from Fig. 4, the highest value reached when extracted for 20 min. When the extraction time was longer than 20 min, the recovery of FP kept almost constant. So, in the following experiments, 20 min was selected for the extraction.

Theoretically, the optimal equilibration temperature for the extraction occurs when temperature is 15–20 °C higher than the cloud-point of surfactant. So the influence of temperature on the extraction efficiency was studied in the range of 45–65 °C.

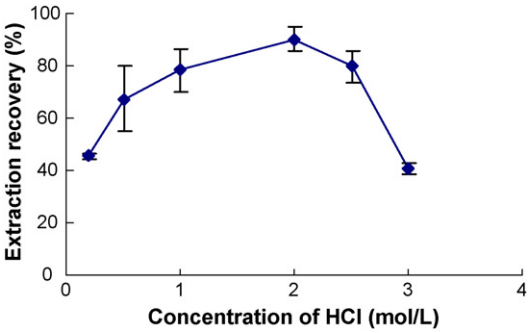

Fig. 3. Effect of concentration of HCl on the extraction efficiency ($n = 3$). Experimental condition: 5% Genapol X-080, 50 °C, 30 min.

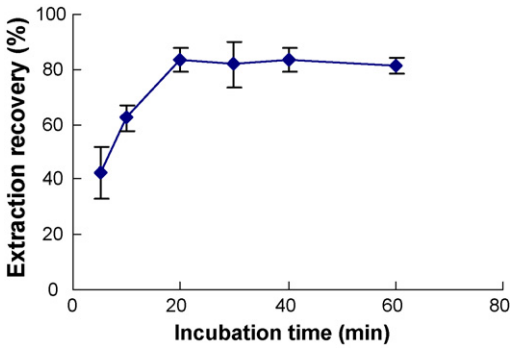

Fig. 4. Effect of incubation time on the extraction efficiency ($n = 3$). Experimental condition: 5% Genapol X-080, 50 °C, with addition of 2 M HCl.

The extraction recovery increased slightly when the temperature ranged from 50 to 60 °C. No significant increase was observed for higher temperatures. Therefore, CPE process was carried out at 50 °C (Fig. 5).

3.2.5. Effect of centrifugation time

In general, centrifugation time hardly affects micelle formation but accelerates phase separation, in the same sense as in conventional separations of a precipitate from its original aqueous environment. The effect of centrifugation time upon extraction efficiency was studied at 3500 rpm in the range of 5–20 min. The complete phase separation was achieved after 5 min. Centrifugation time of 10 min were chosen as optimal, with good efficiency for separating both phases and experimental convenience.

3.3. Method validation

3.3.1. Specificity

The specificity of the method was evaluated by comparing chromatograms obtained from six independent plasma samples from rats, each as a blank and a spiked sample. Typical chromatograms are shown in Fig. 6. The analyte and internal standard were both eluted without any interference from endogenous substances. The retention times for FP and IS were 7.1 and 8.3 min, respectively. And under the described chromatographic conditions, no metabolites of FP were observed in rat plasma. The results showed that the described HPLC method was selective for the determination of FP in rat plasma.

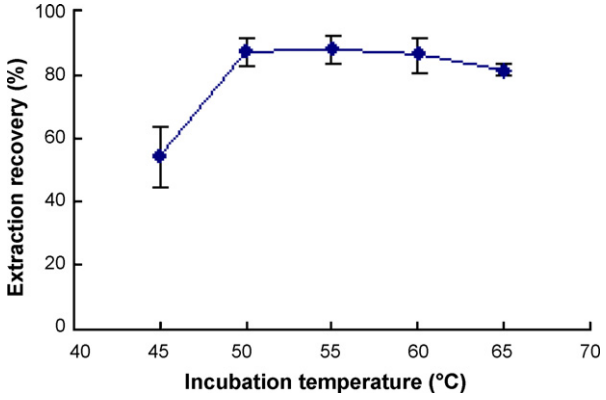

Fig. 5. Effect of incubation temperature on the extraction efficiency ($n = 3$). Experimental condition: 5% Genapol X-080, 20 min, with addition of 2M HCl.

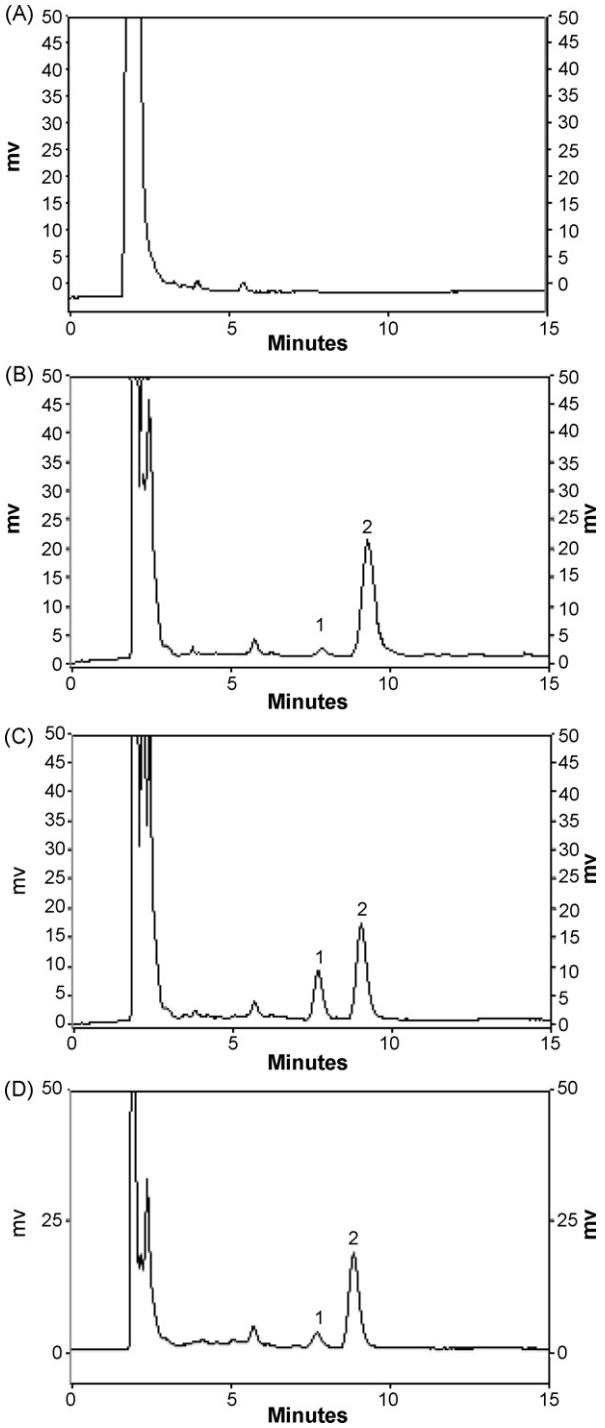

Fig. 6. Typical chromatograms for determination of FP in plasma samples, (A) blank,(B) spiked sample with FP of 0.1 μg/ml, (C) spiked sample with FP of 40 μg/ml and (D) plasma sample from rat 0.5 h after transdermal administration. Peak identification: 1 = FP, 2 = IS.

3.3.2. Linearity

The linearity of each calibration curve was determined by plotting the peak-area ratio (y) of FP against internal standard versus the nominal concentration (x) of FP. The calibration curves were obtained by weighted ($1/x^2$) linear regression analysis. The plotted calibration curves and correlation coefficients >0.99 confirmed that the calibration curves were linear over the concentration range 0.2–50.0 and 0.1–10.0 μg/ml for FP after oral and transder-

Table 1
Precision, accuracy and recovery of flurbiprofen assay in rat plasma

	Concentration (μg/ml)	Precision				Accuracy (R.E.%)	Recovery (Mean ± S.D.) (%)
		Intra-day (n = 6)		Inter-day (n = 18)			
		Mean ± S.D. (μg/ml)	R.S.D. (%)	Mean ± S.D. (μg/ml)	R.S.D. (%)		
Oral	0.5	0.48 ± 0.02	3.3	0.49 ± 0.03	5.3	−1.9	87.7 ± 6.0
	10	10.10 ± 0.35	3.5	10.20 ± 0.52	5.1	2.0	84.5 ± 1.6
	40	42.56 ± 0.86	2.1	41.52 ± 2.18	5.3	3.8	90.6 ± 0.6
Transdermal	0.25	0.25 ± 0.01	3.9	0.25 ± 0.02	10.1	−1.7	86.6 ± 1.7
	2.5	2.44 ± 0.10	4.0	2.44 ± 0.19	8.0	−2.4	85.1 ± 1.5
	8.0	7.80 ± 0.12	1.5	7.88 ± 0.64	8.1	−1.5	88.7 ± 1.4

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

Table 2
Stability of flurbiprofen in rat plasma (n = 3)

	Theoretical concentration (μg/ml)					
	Oral			Transdermal		
	0.5	10	40	0.25	2.5	8
Plasma stored at room temperature for 4 h						
(Mean ± S.D. (μg/ml)	0.48 ± 0.02	9.61 ± 0.28	39.88 ± 1.26	0.24 ± 0.00	2.36 ± 0.07	7.69 ± 0.06
R.S.D. (%)	4.26	2.95	3.15	1.05	2.80	0.83
R.E. (%)	−4.1	−0.6	3.2	−6.0	−5.5	−3.9
Post-preparative stability (storage at 4 °C for 24 h)						
Mean ± S.D.	0.48 ± 0.02	10.15 ± 0.34	41.08 ± 1.39	0.23 ± 0.00	2.42 ± 0.05	7.48 ± 0.06
R.S.D. (%)	3.18	3.35	3.39	0.35	2.19	0.84
R.E. (%)	−3.2	1.5	2.7	−7.2	−3.3	−6.5
Long-term stability (storage at −20 °C for 14 days)						
Mean ± S.D. (μg/ml)	0.48 ± 0.02	10.04 ± 0.39	41.90 ± 0.95	0.23 ± 0.00	2.65 ± 0.04	8.27 ± 0.13
R.S.D. (%)	4.07	3.84	2.28	1.93	1.67	1.52
R.E. (%)	−4.61	0.44	4.76	−6.2	6.1	3.4
Three freeze and thaw cycles						
Mean ± S.D. (μg/ml)	0.49 ± 0.02	10.36 ± 0.25	41.22 ± 1.30	0.25 ± 0.00	2.44 ± 0.05	7.58 ± 0.19
R.S.D. (%)	3.23	2.43	3.14	2.02	2.17	2.53
R.E. (%)	−1.8	3.6	3.1	−1.9	−2.3	−5.2

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

mal administration, respectively. Typical standard curve were as follows, oral: $y = 5.99 \times 10^{-2}x + 9.39 \times 10^{-2}$ (r = 0.997); transdermal: $y = 1.67 \times 10^{-2}x + 8.68 \times 10^{-2}$ (r = 0.998).

3.3.3. Limit of quantification

The LLOQ for determination of FP in rat plasma, defined as the lowest concentration analyzed with an accuracy ≤15% and an precision ≤15%, was found to be 0.1 μg/ml.

3.3.4. Accuracy and precision

The intra- and inter-day precisions and accuracy results are summarized in Table 1. In this study, the intra- and inter-day precisions were less than 10.1%, and the accuracy was within ±3.8% for each QC sample. The obtained values were lower than the limits required for biological sample analysis. These data indicated that the assay was reproducible, accurate and reliable.

3.3.5. Extraction recovery

The results (Table 1) showed that extraction recoveries of FP from rat plasma, for oral administration, were 87.7 ± 6.0, 84.5 ± 1.6 and 90.7 ± 0.6% at concentrations of 0.5, 10 and 40 μg/ml, respectively. For transdermal administration, the recoveries determined at 0.25, 2.5 and 8 μg/ml were 86.6 ± 1.7, 85.1 ± 1.5 and 88.7 ± 1.4%, respectively. For the internal standard (80 μg/ml), the mean extraction recovery was 84.4 ± 4.8%.

3.3.6. Stability

The stability of FP in plasma was evaluated by analyzing spiked quality control samples. The stability data was summarized in Table 2, which indicated FP was stable under different conditions.

3.4. Pharmacokinetic study of flurbiprofen in rats

The method yielded satisfactory results for determination of FP in rat plasma and has been successfully applied to the pharmacokinetic studies of FP after oral and transdermal administration to rats. The mean plasma concentration-time profiles are shown in Fig. 7 and its pharmacokinetic data are listed in Table 3.

It was shown that the pharmacokinetic parameters of FP in rat after oral and transdermal administration of the same dosage (10 mg/kg) were very different. Compared with oral administration, the maximum plasma concentration (C_{max}) of FP after transder-

Table 3
Pharmacokinetic data of FP in rats (n = 6)

Parameter	Estimate (mean ± S.D.)	
	Oral	Transdermal
T_{max} (h)	2.33 ± 1.37	3.67 ± 0.82
C_{max} (μg/ml)	29.44 ± 1.91	2.49 ± 0.59
$t_{1/2}$ (h)	4.15 ± 0.54	10.12 ± 3.83
AUC_{0-t} (μg h/ml)	240.05 ± 41.63	29.16 ± 4.52
$AUC_{0-\infty}$ (μg h/ml)	244.89 ± 40.89	34.43 ± 6.18

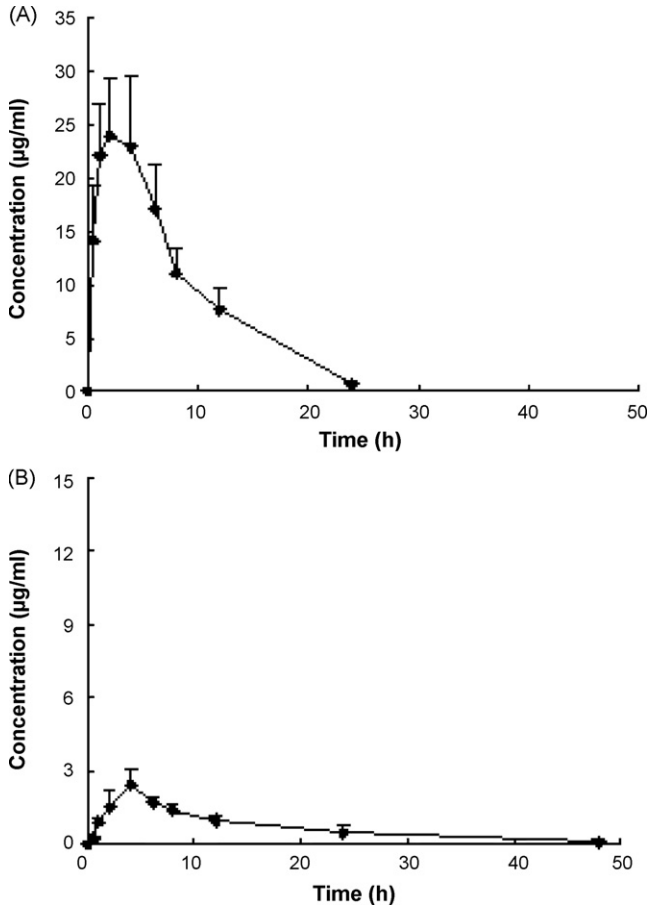

Fig. 7. Mean plasma concentration-time curve for FP in rat plasma after oral (A) and transdermal (B) administration (each point and bar represents the mean ± S.D., $n = 6$).

mal administration was diminished obviously, the time to reach the maximum plasma concentration (T_{max}) and the apparent elimination half-life ($t_{1/2}$) were all prolonged. The AUC_{0-t} value of FP after oral administration was 242.28 ± 42.42 μg h/ml, which was about 9.8-fold variance compared with that (24.67 ± 2.58 μg h/ml) obtained after transdermal administration. These results indicated that the NLC-gel formulation could decrease the plasma concentration of FP, which suggested that FP in NLC-gel might mostly present in rat skin, muscle or joint. Thus, FP NLC-gel could avoid or decrease gastrointestinal irritation and the systemic toxicity produced by FP after oral administration. Our future work will aim at further investigations on the drug concentration in rat skin, muscle and joint after topical administration of FP NLC-gel.

4. Conclusion

The cloud point extraction technique was applied as an effective method for the extraction and preconcentration of FP from plasma samples. Coupled with HPLC–UV detection, the method has been proved to be simple, rapid, sensitive, accurate and reliable for assessment of FP in biological samples and pharmacokinetic studies for clinical purpose.

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