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Short communication

An improved HPLC method with fluorescence detection for the determination of pyrene in rat plasma and its pharmacokinetics

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ABSTRACT

A high-performance liquid chromatographic method with fluorescence detection (HPLC–FLD) for the determination of pyrene in rat plasma was developed and validated. The method used fluorene as internal standard (IS), following a single-step protein precipitation, the analyte and internal standard were separated on a C18 column with a mobile phase containing methanol–water (90:10, v/v) at a flow rate of 1 ml/min. The analytes were detected by using fluorescence detection at an excitation and emission wavelength of 265 and 394 nm, respectively. Two calibration curves were constructed in the range of 2–100 ng/ml and 0.1–5 μg/ml for pyrene with a lower limit of quantitation (LLOQ) of 2 ng/ml. Both intra-day and inter-day precision were less than 6% except at LLOQ, for which the precision was 10.6 and 9.8, respectively. Accuracy ranged from 98.3 to 103.6%, except at LLOQ, for which the accuracy was about 85%. The recovery ranged from 84.7 to 95.0% at the low, medium and high concentrations. The present HPLC–FLD method was rapid, sensitive, and reliable. The method described herein had been successfully applied for the pharmacokinetic studies in female Wistar rats after administration of 10 mg equivalent pyrene/kg dose of solution of pyrene and 1 mg equivalent pyrene/kg dose of pyrene-loaded nanoparticle.

1. Introduction

Recently, colloidal drug delivery systems like nanoparticles of biodegradable polymers have attracted great interests and have been extensively investigated. The advantages of such a formulation include good biocompatibility, reduced systemic side effects, high capability to cross some physiological barriers, sustained and controlled release drug delivery as well as targeted delivery of the drug [1–4]. The in vitro and in vivo study, especially pharmacokinetics study of the nanocarrier is imperative to accurately portray nanoparticle effects [5]. Towards above object, the fluorescence probe technique is widely used. 6-Coumarin and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine were applied to study cell uptake and biodistribution of the nanoparticles [6,7]. Tyrphostin AG-1295 and Prodan were used to investigate the release mechanism of the nanoparticle [8,9]. PLA-pyrene, 3,3′-dioctadecyloxacarbo-cyanine were incorporated as a fluorescent dye into biodegradable nanoparticles for the research of the carriers [10,11]. However, some fluorescent labels are restricted in biological analysis fields because of some limitations, such as the low detection sensitivity, having no suitable IS for the quantification.

Pyrene (Fig. 1a), a polycyclic aromatic hydrocarbon, has a long singlet lifetime, and the vibrational band structure of its emission is sensitive to the environment, so it is by far the most frequently used dye in the determination of critical micelle concentration for colloidal drug delivery systems [12]. Due to its extreme hydrophobicity and good fluorescence properties, pyrene could be used as a model hydrophobic drug and be incorporated into nanoparticles for the pharmacokinetics study. Gao provided a modified method for preparation of pyrene-loaded polystyrene nanoparticles [12]. Zhang incorporated pyrene into methoxy polyethylene glycol–polylactide (MPEG–PLA) nanoparticle to study the transport pathway of the nanoparticle from nasal cavity to brain after nasal administration [13], but nearly no report has been published for the pharmacokinetics of pyrene-loaded nanoparticles, which may provide a strategy for the design of the polymeric nanoparticles.

Although few methods have been reported for the quantification of pyrene in plasma, good methods have been established for the quantitative determination for 1-hydroxypyrene [14–16], which is commonly measured as biomarkers to assess exposure of human to polycyclic aromatic hydrocarbons. The pharmacokinetics, tissue distribution and elimination of pyrene were investigated in rats [17,18]. To extract pyrene from biological samples, the analytical methods involved multiple steps of liquid–liquid extraction, which used relatively toxical agents such as hexane, followed by evaporation to prepare and concentrate the sample prior to injec-

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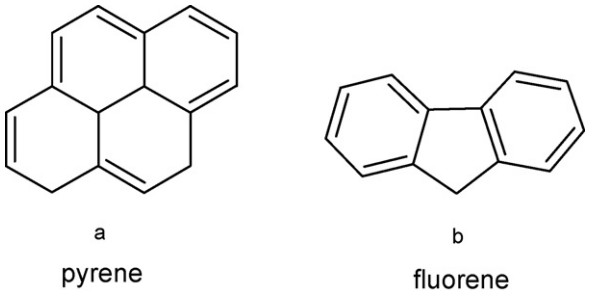

Fig. 1. Chemical structures of pyrene (a) and fluorene (b).

tion to an HPLC system, the pretreatment of the biological samples were laborious and time-consuming. Due to the multiple steps of sample pretreatment and in order to maximize the sensitivity of the assay, a relatively large volume of blood (0.35–0.5 ml) was needed. Diode array detector at 230 nm was used to measure the processed sample [18], which may not be sensitive for determination of trace samples. Additionally, the chromatographic condition of both methods was not suitable for long-term use because of large flow rate (1.5 ml/min). To the authors' best knowledge there was no entirely validated HPLC method reported in the literature for quantification of pyrene in biological sample, so the aim of the current study was (1) to develop a rapid and sensitive HPLC method for the determination of pyrene concentration in plasma and its validation; (2) to apply the established method to preliminarily study the pharmacokinetics of pyrene solution and pyrene-loaded nanoparticle. Herein, we report an HPLC method with fluorescence detection for analysis of pyrene in biological samples using fluorene (Fig. 1b) as internal standard that outperformed both sensitivity and reproducibility. This method had been comprehensively validated, offering the advantage of simplicity with adequate sensitivity, selectivity, precision and accuracy for the determination of pyrene, while very small volume plasma was needed. The assay was also successfully applied in the pharmacokinetic studies of pyrene in female Wistar rats. The development of the method would facilitate the ease of adaptability of pyrene assay in other biological samples such as urine, human plasma and so on.

2. Experimental

2.1. Chemicals and reagents

Pyrene was purchased from Fluka (purity >99%, Switzerland). Fluorene was obtained from Alfa Aesar Corp. (purity >98%, Tianjin, China). Polyoxyethylene hydrogenated castor oil was kindly provided by BASF Corp. (Ludwighshafen, Germany). HPLC-grade methanol was from Hebei Handan reagent Co. Ltd. (Hebei, China). Ultrapure water was purified by HFSuper-NW series from Heal Force Development Ltd. (Hong Kong, China). Other chemicals were of analytical grade. Pyrene-loaded MPEG–PLA nanoparticle suspension was prepared by spontaneous emulsification solvent diffusion (SESD) method in the laboratory.

2.2. Chromatographic conditions

The integrated high-performance liquid chromatography system Agilent 1100 series (USA) were equipped with a quaternary pump, a degasser, an autosampler, a column thermostat, and a violet-visible detector. A Waters 474 fluorescence detector (USA) was coupled to the Agilent system. Chromatographic separation was performed using an analytical hypersil C18 column (5 μm, 250 mm × 4.6 mm) (Dalian Yilite Technologies Inc., Dalian, China) with a column temperature of 25 °C. The mobile phase consisted of a mixture of methanol–water (90:10, v/v) and was filtered using a 0.45 μm filter in a solvent filtration apparatus. After degassing by ultrasonication the mobile phase was delivered at a flow rate of 1 ml/min. The column effluent was monitored by a UV detector and a fluorescence detector, for the UV detector the detection wavelength was 240 nm, while the fluorescence detector was set at an excitation wavelength of 265 nm and an emission wavelength of 394 nm.

2.3. Calibration standards and quality control samples

Primary standard stock solutions of pyrene (1 mg/ml) and fluorene (360 μg/ml) were prepared in methanol. Working solutions of pyrene were prepared by appropriate dilution with methanol just before use, using amber glass volumetric flasks in order to avoid photo degradation. The IS working solutions of 36 μg/ml were prepared in the same manner. All solutions were stored in darkness at 4 °C.

Five microliter aliquots of pyrene working solutions were added to 50 μl drug-free rat plasma to obtain pyrene calibration standards (2, 5, 10, 20, 40, 80, 100 ng/ml and 0.1, 0.2, 0.5, 1, 2, 5 μg/ml) in plasma samples for two calibration curves. Quality control (QC) samples were separately prepared in the similar manner for two calibration curves, the range of the two calibration curves were 2–100 ng/ml and 0.1–5 μg/ml, respectively. For calibration curve one, 2, 50, and 100 ng/ml plasma samples were corresponding to the low QC, medium QC, and high QC, respectively, the concentration of low QC was the same as LLOQ. For another calibration curve, 0.2, 2.5, and 5 μg/ml plasma samples were corresponding to the low QC, medium QC, and high QC, respectively.

2.4. Sample pretreatment

A 50 μl volume of plasma standard or sample was transferred to a 1.5 ml centrifuge tube, and then 5 μl of IS working solution (36 μg ml^{-1}) was spiked and vortex mixed for several seconds. Next 200 μl aliquot of methanol was added and the sample was vortex-mixed for 2 min. After vortex mixing, the mixture was centrifuged at 10,000 rpm for 10 min at a low temperature (4 °C). At last, 50 μl of the supernatant were injected into the HPLC for analysis.

2.5. Bioanalytical method validation

2.5.1. Specificity

Specificity of the method was assessed by analysis five independent sources of blank rat plasma or plasma samples spiked with pyrene and fluorene, observing the extent to which interferent from plasma may interfere with the analyte or the internal standard. As 1-hydroxypyrene is a phase I metabolite of the pyrene, its potential interference with the analytes was also considered.

2.5.2. Calibration curves and linearity

Every calibration standard concentration was triplicate. After injecting all the processed calibration standard samples of various concentrations covering the working range of the assay, two calibration curves were established in the range of 2–100 ng/ml and 0.1–5 μg/ml. The calibration curves were generated by plotting peak area ratios of analytes to IS against the respective standard concentrations. The acceptance criteria for a calibration curve was (1) correlation coefficient (r^2) was greater than or equal to 0.99, (2) each back-calculated standard concentration must be within 15% deviation from the nominal value except at the LLOQ, for which the maximum acceptable deviation was set at 20%.

2.5.3. Determination of the lower limit of quantitation and limit of detection

Batches of blank rat plasma were spiked with two different concentrations (1 and 2 ng/ml) of pyrene and measured to determine the LLOQ which is defined as a lowest concentration of analyte that can be determined with acceptable precision and accuracy under stated experimental conditions. LLOQ was identified based on the two criteria [19]: (1) the lowest concentration of the analyte that produced S/N ratio of greater than 10; (2) the analyte response that can be determined with sufficient precision and accuracy, i.e. the percentage deviation and %R.S.D. are to be less than 20%. The lowest concentration that meets both the mentioned criteria was accepted as LLOQ.

The limit of detection (LOD) for the determination of pyrene in the proposed assay was established by analysis of signal-to-noise ratio (S/N ratio) which was obtained by serial extraction of plasma samples spiked with decreasing concentrations of pyrene. The analyte concentration that produced S/N ratio greater than 3 was accepted as LOD.

2.5.4. Extraction recoveries

The recovery of pyrene from plasma was determined by injecting the processed QC samples at three concentrations of low QC, medium QC and high QC for two calibrations. Recovery was evaluated by comparing the analyte peak areas obtained from the QC samples ($n = 5$) after extraction with those obtained from the corresponding unextracted reference standards prepared at the same concentrations, respectively. Recovery of IS was evaluated by comparing the mean peak areas of five extracted quality control samples with mean peak areas of five reference solutions (unprocessed) of the same concentration.

2.5.5. Accuracy and precision

Intra-day accuracy and precision evaluations were performed by repeated analysis of pyrene in rat plasma on the same day. The run consisted of a calibration curve plus five replicates of each low, medium and high QC samples. Inter-day accuracy and precision were assessed by analysis of samples consisting of a calibration curve and five replicates of three concentrations samples for pyrene on three consecutive days.

Accuracy was calculated as the percentage of the concentration of drug measured from calibration curve to the theoretical concentration value of drug added to the blank plasma. Precision was expressed as the percentage coefficient variation, C.V. (%), of measured concentrations for each QC samples. The values within ±15% for accuracy and precision were considered acceptable, except for concentrations at the LLOQ, where 20% was accepted.

2.5.6. Stability

The stability of the stock solutions and working solutions of pyrene and fluorene, stored at 4 °C for 1 month was tested by comparing the instrument responses with that of freshly prepared solutions at the same concentration. The stability experiments of pyrene in rat plasma were carried out on three conditions: after three freeze–thaw cycles, after storage at room temperature for 24 h and at −20 °C for 1 month. The stability of pyrene in rat plasma was investigated using five replicates of each low, medium and high QC samples. The stability was determined by comparing the calculated concentration of test samples with the nominal concentration of pyrene. Twenty-four hours autosampler stability of plasma QC samples was also tested.

2.5.7. Ruggedness

For ruggedness studies, another different reversed-phase column, LiChrospher C18 (5 μm, 250 mm × 4.6 mm, Jiangshu, China) was used. The samples of blank human plasma spiked with pyrene working solution were also pretreated according to the established method in rat plasma, specificity and recovery were investigated.

2.5.8. Application to pharmacokinetics study of pyrene

The pharmacokinetics study was conducted in accordance with the ethical guidelines for investigations in laboratory animals and approved by the Ethical Committee of Huazhong University of Science and Technology. Three female Wistar rats, which were obtained from Hubei Center for Disease Control and Prevention (Wuhan, China) were fasted overnight and had free access to water throughout the experimental period. The formulation used for administration was a solution in 25% polyoxyethylene hydrogenated castor oil. Rats were injected intravenously through the tail vein at a dose of 10 mg equivalent pyrene/kg weight of body. Blood samples about 0.3 ml were collected from the retro-orbital plexus at designated time points (0.5, 1, 2, 3, 4, 6, 8, 10, 12, 24 and 48 h) into microcentrifuge tubes containing heparin sodium. Plasma was obtained by centrifuging the blood at 4000 rpm for 10 min and was separated into clean tubes and frozen at −20 °C until assay. Pyrene plasma concentration levels were determined using the present HPLC method. New calibration curve and QC samples were done when determining a batch of plasma samples.

In order to further test the sensitivity of the developed method, pyrene-loaded MPEG–PLA nanoparticle suspension was injected intravenously at a low dose of 1 mg equivalent pyrene/kg weight of body.

Pharmacokinetics analysis was carried out by noncompartmental method with the aid of the software DAS2.0 (issued by the State Food and Drug Administration of China for pharmacokinetic study), and pharmacokinetic parameters were obtained at the same time.

3. Results and discussion

3.1. Optimization of the chromatographic conditions

Methanol and water along with acetonitrile and water systems are often used as mobile phase in the reverse chromatography. As benzene and naphthalene are usually separated by the mobile phase composed of methanol and water and pyrene is a neutral molecule, we chose methanol and water without any additive as mobile phase instead of acetonitrile and water, moreover, methanol is cheaper than acetonitrile.

Several trials to screen the ratios of methanol to water were carried out in order to obtain good peak shape and high theoretical plates, and reasonable retention time was also needed to considerate. Sometimes the change of the ratio may significantly affect the response of the analytes. Most important, the analyte and IS should be separate from the interferences of biomatrix components like plasma proteins at baseline. The chromatographic characteristics obtained under different ratios are shown in Table 1. From the

Table 1
The chromatographic characteristics obtained under different ratios of methanol to water

Ratio	Analyte	Retention time (min)	Plate number	Symmetry	Resolution
95:5	Pyrene	6.315	12,443	0.78	–
	Fluorene	4.898	11,031	0.83	7.12
90:10	Pyrene	8.315	11,884	0.87	–
	Fluorene	6.012	11,150	0.86	9.07
80:20	Pyrene	16.815	11,282	0.84	–
	Fluorene	10.207	11,185	0.85	13.12

table, it can be seen that, when the ratio of methanol to water was 90:10, two sharpened and symmetrical peaks of pyrene and fluorene were obtained, the column efficiency were 11,884 and 11,150, respectively. The effective separation was achieved between the analyte and IS which were also completely separated from the interferences. A short run time is obtained because the retention time of pyrene and fluorene were 8.2 and 6.0 min, respectively, and the total run was 15 min, thus we can analyze more samples in a working day. The peak height rose with the increasing content of methanol, but the improvement was not significant, when the ratio was 95:5, sometimes, the IS could not be separated from the polar substance generated by plasma. Finally, the ratio of methanol to water was set to 90:10.

The injection volume of the sample was also investigated. 20, 50, and 100 μl volume of processed sample were injected into the HPLC system sequentially. It was found that when injection volume increased from 20 to 50 μl, no significant reducing column performance was observed and the peak shape was still sharp and symmetrical, but the plate number obviously decreased with bad peak shape when injection volume increased from 50 to 100 μl. In order to improve the sensitivity and prolong the life of the column, 50 μl injection volume was chosen.

3.2. Sample pretreatment

First, the selection of the IS must be solved. We had used the structural analogue of pyrene such as 9-carboxylic acid anthracene and anthracenone as IS, neither good peak shape nor proper retention time was obtained. Fluorene was selected as an IS because of relatively high and reproducible recovery and suitable retention time. In addition, the use of fluorene offered satisfactory validation results of the developed method. The present study was the first report to apply fluorene as an internal standard for HPLC analysis of pyrene. Conventional extraction procedures including protein precipitation and liquid–liquid extraction were studied. Ethyl acetate, *n*-heptane, dichloromethane and chloroform were used to extract pyrene and fluorene from the plasma. After the extraction, the organic layer was transferred to a 1.5 ml Eppendorf tube and evaporated to dryness under a stream of nitrogen in a water bath at 25 °C. The residues were reconstituted in proper volume mobile phase. If these solvents were directly injected into the HPLC system, high background noise would affect the baseline, besides, chronically using dichloromethane and chloroform would damage the system and be harmful to human health. The process of the sample concentration was time-consuming, so protein precipitate method was considerated. Perchloric acid, methanol, ethanol, acetone and acetonitrile were used to precipitate the plasma protein, and then the upper layer was directly injected for analysis. There was bare response detected in the sample processed by perchloric acid, the extreme low solubility of pyrene in water was the possible reason. The extraction efficiency had no obvious difference between methanol and acetonitrile and was higher than that of ethanol and acetone; furthermore, 90% of the mobile phase was methanol. Finally, we chose methanol as the reagent for plasma sample pretreatment. Extraction time and volume of methanol were tested, and the results indicated that extraction recoveries were not improved when the shaking time was longer than 2 min and volumes larger than 200 μl. Furthermore, unlike the traditional HPLC methods that require at least 100 μl and even more volume blood of sample for the experiment [17,18], this method used only 50 μl volume of sample, which facilitated the collection of the blood in the period of pharmacokinetics study and resulted in a less consumption of extraction solvents and shorter sample pretreatment time.

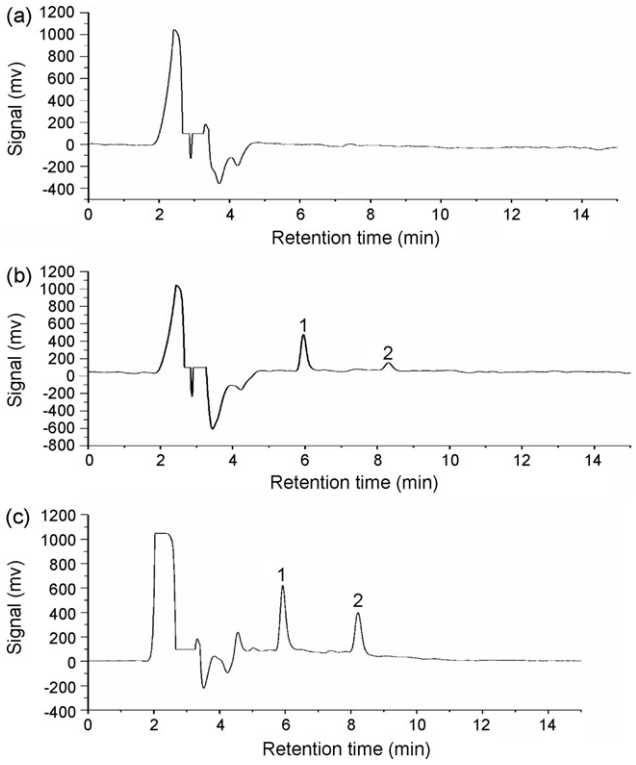

Fig. 2. Representative chromatograms of (a) drug free, blank rat plasma; (b) blank rat plasma spiked with pyrene at low concentration (2 ng/ml) and 3.6 μg/ml of IS and (c) a plasma sample obtained at 2 h from rat dosed with pyrene at 10 mg/kg by intravenous injection. Peak 1, fluorene (internal standard); Peak 2, pyrene.

3.3. Method validation

3.3.1. Specificity

Fig. 2 shows the representative chromatograms of blank plasma, blank plasma samples spiked with pyrene at 2 ng/ml and plasma sample obtained from a rat following an injection 10 mg/kg dose of pyrene. The retention time of IS and pyrene were 6.0 and 8.2 min, respectively. As described above, good resolution was achieved between analyte and IS and no interference from several different sources of rat plasma was observed interfering the separation and quantitation of pyrene. In the real pharmacokinetic study samples, 1-hydroxypyrene did not interfere with the determination of the analytes.

3.3.2. Linearity, limit of detection and lower limit of quantitation

The calibration model for the two calibration curves was selected based on the analysis of the data by linear regression and with weighting factors ($1/x$, $1/x^2$ and 1). The residuals were slightly improved by weighted ($1/x^2$) least-squares linear regression for calibration curve in the range of 0.1–5 μg/ml. For calibration curve in the range 2–100 ng/ml, the weighted factor was set to 1.The peak area ratio of pyrene to IS in rat plasma was linear with respect to the analyte concentration over the range 2–100 ng/ml and 0.1–5 μg/ml. The regression equation for calibration one was $Y = 0.0176X + 0.0384$ (correlation coefficient, $r = 0.999$), and $Y = 2.020X + 0.125$ (correlation coefficient, $r = 0.998$) was the regression equation for another calibration curve in the range 0.1–5 μg/ml, where Y is the mean peak area ratio of the analyte to the IS and X is the concentration of the analyte. Each back-calculated standard concentration was within 10% deviation from the nominal value except at LLOQ, for which the deviation was 18.2. The LOD for pyrene was found to be 0.5 ng/ml (S/N ≥ 3) and LLOQ was 2 ng/ml with acceptable preci-

Table 2
Intra-day and inter-day accuracy and precision of pyrene in rat plasma ($n = 5$)

Nominal concentration (ng/ml)	Intra-day			Inter-day		
	Mean ± S.D. calculated (ng/ml)	Recovery (%)	R.S.D. (%)	Mean ± S.D. calculated (ng/ml)	Recovery (%)	R.S.D. (%)
2	1.68 ± 0.178	84.0	10.6	1.72 ± 0.169	86.0	9.80
50	50.25 ± 1.89	100.5	3.78	48.79 ± 2.74	97.6	5.60
100	99.20 ± 4.16	99.2	4.19	98.34 ± 3.23	98.3	3.28
200	203.5 ± 7.80	101.8	3.84	201.7 ± 6.9	100.9	3.42
2500	2590 ± 133.6	103.6	5.16	2467 ± 143.6	98.7	5.82
5000	5073 ± 301.0	101.5	5.93	5022 ± 299.7	100.4	5.97

Table 3
Extraction recoveries of pyrene from rat plasma ($n = 5$)

Nominal concentration (ng/ml)	Mean ± S.D. recovery (%)	R.S.D. (%)
2	84.7 ± 1.4	1.6
50	92.3 ± 1.4	1.5
100	92.9 ± 2.1	2.3
200	95.0 ± 3.7	3.8
2500	88.8 ± 3.3	3.7
5000	86.3 ± 1.8	2.1

sion and accuracy using 50 μl plasma sample. The sensitivity could be increased by using more volume of plasma or concentrating the processed sample, but the presented method was sensitive enough for the pharmacokinetics study.

3.3.3. Accuracy and precision

The intra-day and inter-day accuracy and precision values of the method are presented in Table 2. The intra-day coefficient of variation (%R.S.D.) for pyrene ranged from 3.78 to 10.60% and the accuracy from 84.0 to 103.6%. The inter-day %R.S.D. for the analyte was from 3.28 to 9.80% and the accuracy from 86 to 100.9%. The results indicated that the assay was reproducible, accurate and reliable.

3.3.4. Extraction efficiency

The recovery of pyrene was higher than 80% at all the six concentrations studied (Table 3) and the extraction efficiency did not show obvious dependent relation with concentration. From the obtained results, it could be concluded that the volume of the methanol was sufficient to extract the analyte. The recovery of the IS was about 80%, relatively lower than that of pyrene, but stable in all the samples, which was important in the routine analysis.

3.3.5. Stability

The stock solution of pyrene was stable at 4 °C in the investigation period since the response of the analyte was found to be within 99.0–101% of that of the freshly prepared solutions with the same concentration (data not shown). The IS was also stable at 4 °C for one month. The stability data of pyrene in rat plasma on three conditions are shown in Table 4. As shown in Table 4, no significant reduction of pyrene content in the rat plasma was observed under any of those conditions. The processed samples in the autosample tray showed no obvious concentration change for 24 h.

3.3.6. Ruggedness

The processed QC samples were successfully separated on a LiChrospher C18 (5 μm, 250 mm × 4.6 mm, Hanbang, China) column with good peak shape and resolution in the established chromatographic conditions, only a slightly delay of the retention time was observed. It could be predicted that the existing chromatographic conditions could be used in several kinds of C18 column. The sample pretreatment method was used in the samples of blank human plasma spiked with pyrene working solution, and there was good baseline separation of pyrene, IS and the interference from human plasma. Recovery of the method in human plasma was also high as that in rat plasma. From the above results, we can anticipate that application of the method for monitoring the plasma concentration in human is promising.

3.4. Application to pharmacokinetics study of pyrene

The applicability of the assay method was demonstrated in a pharmacokinetic study of pyrene solution containing surfactant and pyrene-loaded nanoparticle in female Wistar rats. After administration, the plasma concentrations of pyrene were determined by the described method. The pyrene plasma concentration–time data were analyzed by noncompartmental analysis with the aid of the program DAS2.0. Mean (±S.D.) plasma concentration–time profile of pyrene following intravenous injection of solution was depicted in Fig. 3a. The profile revealed that the elimination of the pyrene was very rapid, but the concentration was detectable until 24 h. The pharmacokinetic parameters were summarized in Table 5. Another plasma concentration–time curve which was presented in Fig. 3b was obtained after administration of pyrene-loaded nanoparticle suspension by intravenous injection, all the concentration were above the LLOQ (2 ng/ml) in the entire experimental period until 72 h. Compared to the pharmacokinetics behavior of the solution, the nanoparticle showed a long circulating property, which

Table 4
Stability of pyrene in rat plasma on different conditions ($n = 5$)

Experimental conditions	Parameter	QC concentrations (ng/ml)					
		2	50	100	200	2500	5000
After three freeze–thaw cycles	Mean calculated concentration (ng/ml)	1.71	51.6	94.2	194	2357	4738
	R.S.D. (%)	2.3	3.7	6.5	7.2	6.3	2.1
At room temperature for 24 h	Mean calculated concentration (ng/ml)	1.78	46.7	96.1	190	2432	4863
	R.S.D. (%)	2.7	2.2	1.6	6.2	5.5	2.4
At −20 °C for 1 month	Mean calculated concentration (ng/ml)	1.89	48.7	101.5	201	2478	4976
	R.S.D. (%)	3.6	2.0	4.6	2.7	8.2	3.4
On autosampler for 24 h	Mean calculated concentration (ng/ml)	1.92	50.0	101.4	205	2535	5045
	R.S.D. (%)	2.5	2.4	7.5	3.3	7.1	4.8

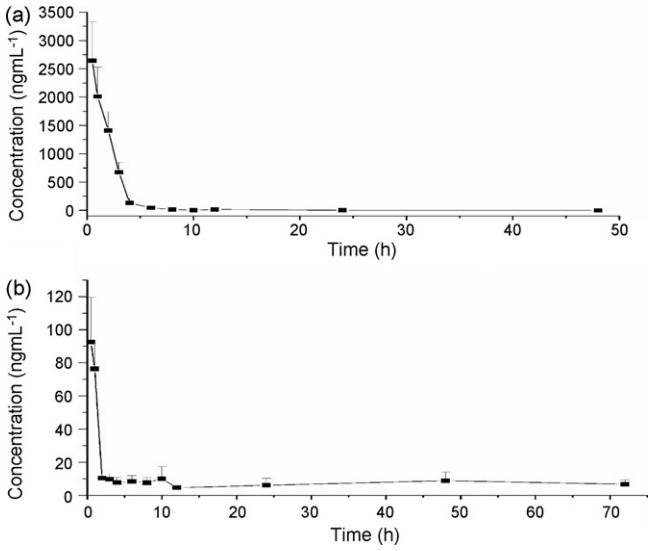

Fig. 3. Mean (±S.D.) plasma concentration–time profile of pyrene in female Wistar rats ($n = 3$), (a) female Wistar rats were administered by intravenous injection of a solution at a dose of 10 mg of pyrene per kg of body weight; (b) female Wistar rats were administered by intravenous injection of a pyrene-loaded nanoparticle suspension at a dose of 1 mg of pyrene per kg of body weight. Some error bars are too small to be shown.

Table 5
Pharmacokinetic parameters obtained after administration by intravenous injection of a solution at a dose of 10 mg of pyrene and a pyrene-loaded nanoparticle suspension at a dose of 1 mg of pyrene per kg of body weight

Parameters	Solution of pyrene	Pyrene-loaded nanoparticle
$MRT_{(0\rightarrow t)}$ (h)	1.656	28.584
$MRT_{(0\rightarrow\infty)}$ (h)	1.680	81.924
$t_{1/2}$ (h)	3.301	39.453
V (L kg^{-1})	7.739	38.999
CL (L h^{-1} kg^{-1})	1.625	0.685

had been proved by other studies [20,21]. The mean residence time ($MRT_{0\rightarrow\infty}$) and elimination half-life ($t_{1/2}$) were 81.924 h and 39.453 h, respectively. Other pharmacokinetics parameters were also listed in Table 5.

4. Conclusion

In the present study, the aim proposed in the introduction was achieved. A novel sensitive, specific, and reproducible fluorimetric HPLC method for the determination of pyrene in rat plasma was developed in this study. The method was comprehensive validated over a concentration range of 1–100 ng/ml and 0.1–5 μg/ml ($r > 0.998$) and it offered good accuracy and precision. The advantages of the method are summarized as below: (1) Only a small volume (50 μl) of sample was needed; (2) the analysis was rapid with a short time run of 15 min and sensitive with the LLOQ of 2 ng/ml; (3) a simple sample pretreatment with protein precipitation was applied compared to multiple extraction and concentration steps of the previously published methods. Fluorene was used for the first time as an internal standard for pyrene analysis and successfully applied to a quantitation of pyrene in plasma with reproducible recovery and suitable retention time. Our laboratory is actually involved in a study to investigate the pharmacokinetics of pyrene-loaded nanoparticle at relatively higher dose and the established method provides a reliable bioanalytical methodology for it.

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