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Determination of arbidol in rat plasma by HPLC–UV using cloud-point extraction

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Abstract

A method based on cloud-point extraction (CPE) was developed to determine arbidol in rat plasma by high performance liquid chromatography separation and ultraviolet detection (HPLC–UV). The non-ionic surfactant Triton X-114 was chosen as the extract solvent. Variable parameters affecting the CPE efficiency were evaluated and optimized. A Zorbax SB-C_{18} column (4.6 mm i.d. × 150 mm, 5 μm particle size) was used for isocratic elution separation at 40 °C with detection wavelength at 316 nm. Under the optimum conditions, the method was shown to be reproducible and reliable with intraday precision below 6.6%, interday precision below 8.8%, accuracy within ±5.0% and mean extraction recovery more than 89.7%, which were all calculated using a range of spiked samples at three concentrations of 0.2, 2 and 16 μg/ml for arbidol in plasma. The linear range was from 0.08 to 20 μg/ml. After strict validation, the method was successfully applied to the pharmacokinetic study of arbidol in rats after oral and intravenous administration, respectively.

Keywords: Cloud-point extraction; Arbidol; HPLC–UV; Triton X-114; Pharmacokinetics

1. Introduction

Arbidol, ethyl-6-bromo-4-[(dimethylamino)-methyl]-5-hydroxy-1-methyl-2-[(phenylthio)methyl]-indole-3-carboxylate (Fig. 1A), is an antiviral active chemical entity, which can also activate phagocytic activity of macrophages and stimulate some forms of cellular and humoral immunity [1]. In Russia, it has been used for the treatment of influenza for several years. Recently, arbidol has been widely used for the treatment of influenza and some other kinds of respiratory infections because of its fair safety and efficacy [2,3]. As the clinical use of arbidol became common, methods for its quantification in biological fluids have attracted the attention of many investigators [4,5]. But the sample preparation involved in all these experiments was tedious traditional liquid–liquid extraction, which has quite a lot of drawbacks such as the use of large amounts of toxic and flammable organic solvents, the analyte losses during the evaporation of solvents and the unavoidable adsorption of non-polar analytes to glass surfaces. In the last decades, the environmental clean cloud-point extraction method has been proposed as a convenient alternative, which overcomes most of the difficulties mentioned above. Compared to the conventional liquid–liquid extraction, CPE requires a very small amount of surfactants that are relatively friendly to environment. Under appropriate conditions such as temperature, concentration of surfactant and equilibration time, the micellar aqueous solutions prepared with non-ionic surfactants undergo phase separation above a certain temperature known as the cloud-point temperature. During the phase-separation processes, these micellar vesicles, which attract non-polar analytes because of hydrophobic interactions, aggregate into a surfactant-rich phase; the left is the aqueous phase with a diluted surfactant concentration, which approximates to its critical micelle concentration (CMC) [6–9]. After clean-up or dilution, the analyte/surfactant-rich phase permits analysis and quantification of the analytes by techniques such as high performance liquid chromatography, gas chromatography, capillary electrophoresis [10] and liquid chromatography–mass spectrometry [11]. The CPE methodology has recently been applied to the extraction of a wide range of analytes from biological and environmental media, including estrogens, vitamin A, vitamin E, kinds of proteins, as well as metal ions [12–17]. All these indicate that CPE has

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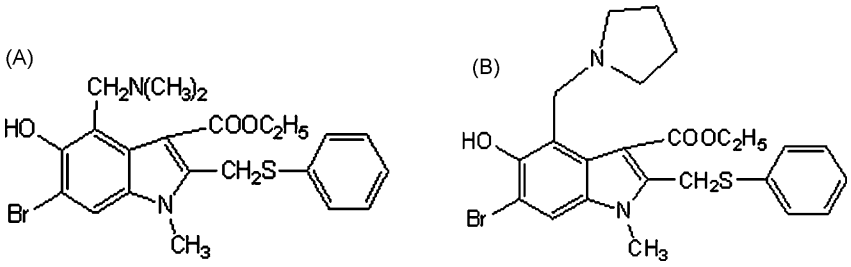

Fig. 1. Chemical structures of arbidol (A) and internal standard (B).

great analytical potential as an effective enrichment method, but reports about its applications on how to extract drugs from plasma for clinical and biomedical purpose were so limited [13,15]. In this paper, we report the quantification of arbidol in rat plasma by CPE preparation using Triton X-114 as the surfactant with HPLC–UV detection to demonstrate the feasibility of CPE in clinical and pre-clinical pharmacokinetic studies.

2. Experimental

2.1. Chemicals and reagents

Arbidol as well as the internal standard, ethyl-6-bromo-4–[(pyrrolyl)-methyl]-5-hydroxy-1-methyl-2-[(phenylthio)methyl]-indole-3-carboxylate (Fig. 1B) (purity >98.0%), was kindly provided by Professor Ping Gong from Shenyang Pharmaceutical University. Stock solutions (2 mg/ml) containing these compounds were prepared by dissolving an appropriate amount of these compounds in acetonitrile. Working solutions were prepared daily by appropriate dilutions of the stock solutions. The non-ionic surfactant Triton X-114 (Acros Organics, New Jersey, USA) was used without further purification. Acetonitrile and phosphoric acid were of HPLC grade. All the other reagents were of analytic grade. Distilled water, prepared with demineralized water, was used throughout the study.

2.2. Instrumentation

The analyses were completed using an Agilent 1100 series HPLC system, including a quaternary pump, a variable wavelength UV detector, a column oven and a Rheodyne 7225i injector. The separation was performed on a Zorbax SB-C_{18} column (4.6 mm i.d. ×150 mm, 5 μm particle size, Agilent Technologies) equilibrated with a mixture of water (0.2% phosphoric acid and 0.1% triethylamine)–acetonitrile (60:40) at a flow rate of 1 ml/min. The detector was operated at 316 nm and the column temperature was maintained at 40 °C. A personal computer equipped with an Agilent Chemstation program for LC systems was used to acquire and process chromatographic data. Peak area was evaluated as the analytical measurement. A thermostatic bath (HH-2, Guohua Medical Instrument Company, PR China), set at the desired temperature, was used to implement cloud point extraction. Centrifugation (TDL-16C, Shanghai Anting Medical Instrumental Factory, PR China) with calibrated centrifugal tubes was applied to accelerate the phase separation process.

2.3. CPE procedure

For the extraction and preconcentration of arbidol, 200 μl of rat plasma sample and 50 μl of internal standard solution (50 μg/ml) were added to a 1.5 ml capped centrifugal tube. To this were added 1 ml of aqueous solution of Triton X-114 at concentration of 5% (w/v) and 100 μl sodium hydroxide solution (1 mol/l). The contents were mixed well with a Vortex Genie Mixture (CAY-1, Beijing Changan Instrumental Factory, PR China) for 5 min, and then incubated in the thermostatic bath at 45 °C for 20 min. The phase separation was then accelerated by centrifugation at 3500 rpm (890 × *g*) for 5 min. After removing of the water phase, a surfactant-rich phase stuck to the bottom 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 100 μl of acetonitrile and centrifugation at 16,000 rpm (18,603 × *g*) for 5 min. Twenty microliter of the upper layer was injected into the HPLC system for analysis.

2.4. Application to pharmacokinetic study

Male Wister rats (220–250 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. Before drug administration, they were fasted overnight. 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.

Two groups of rats, six for each, were given arbidol intravenously (9 mg/kg) and orally (54 mg/kg), respectively. Blood samples (0.5 ml) were collected from the ocular vein before (0 h) and 0.033, 0.083, 0.17, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 9 and 12 h after dosing into heparinized tubes and then immediately centrifuged at 3500 rpm (890 × *g*) for 10 min. The plasma obtained was stored frozen at −20 °C until analysis.

3. Results and discussion

3.1. HPLC–UV conditions

To optimize the chromatographic conditions, the mobile phase system was investigated. The adoption of acidic buffer as a component of mobile phase was found to be essential to minimize the strong interaction of the analytes with the silanol groups

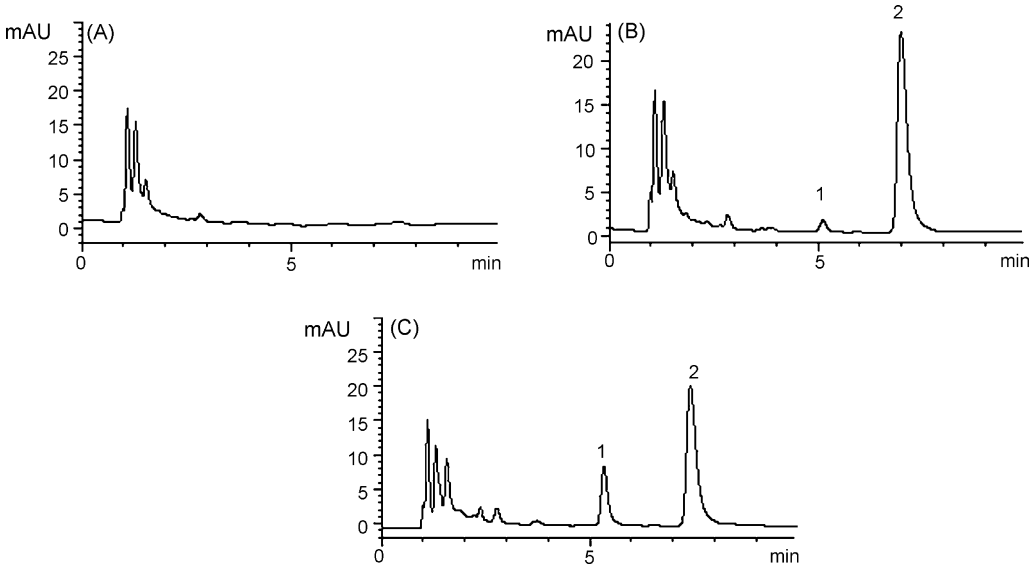

Fig. 2. Typical chromatograms for determination of arbidol in plasma samples. (A) Blank; (B) spiked sample with arbidol of 80 ng/ml; (C) plasma sample from rat 0.17 h after oral administration. Peak identification: 1 = arbidol, 2 = IS.

of column packing, which can also make the peaks symmetric and sharper. Column packed with Zorbax SB-C_{18} material was applied for its sound tolerance of lower pH value and higher oven temperature. Fig. 2 shows the typical chromatograms under these chromatographic conditions.

3.2. Optimization of the extraction process

Triton X-114 was chosen as the CPE surfactant because of its appropriate cloud-point temperature of 23–30 °C and low UV absorbance [10]. The parameters potentially influencing the extraction efficiency (surfactant amount, temperature, pH value and time of incubation) were investigated through the signals of absorbance from the spiked samples of arbidol at concentration of 10 μg/ml in plasma. Three replicates were analyzed to obtain a mean value.

The signals obtained for arbidol as a function of Triton X-114 concentration are shown in Fig. 3. The mean response increased from 39.9 to 144.8 mAU when the concentration of surfactant ranged from 1% to 5%, but decreased as the concentration became higher. 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 course a dilution of the contents, a compromise between recovery and preconcentration has to be adopted when using the cloud-point technique [10]. Percentage of 5% (w/v) Triton X-114 giving rise to about 100 μl of surfactant-rich phase was recommended for its highest extraction efficiency.

Solution pH is an important factor during CPE process involving analytes that possess an acidic or basic moiety. 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 does its neutral form. As a result, a lesser amount of analytes is extracted [9]. In this study, 100 μl of sodium hydroxide (1–2.5 mol/l) was added to the mixture, the effect of which on recovery can hardly be evaluated in the range examined. However, they excelled the procedures carried out under no addition of sodium hydroxide solution—a twice increment in signal response.

Theoretically, the optimal equilibration temperature for the extraction occurs when the temperature is 15–20 °C greater than the cloud-point temperature of surfactant [9]. So the influence of temperature on the extraction efficiency was studied in the range of 35–50 °C. When the temperature became higher (from 35 to 45 °C), the mean signal response increased from 312.2 to 322.6 mAU. No significant increment was observed for higher temperatures. Therefore, CPE process was carried out at 45 °C.

The influence of the incubation time on the ability of Triton X-114 to extract arbidol from plasma was examined in the range between 5 and 30 min (Fig. 4). Extraction time of less then

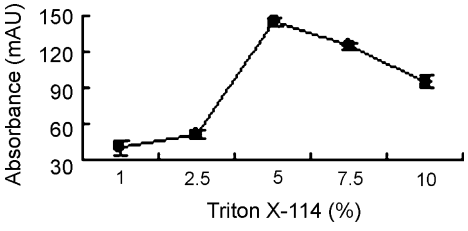

Fig. 3. Extraction effect of arbidol as a function of Triton X-114 concentration. Experimental condition: 45 °C, 20 min, no addition of NaOH.

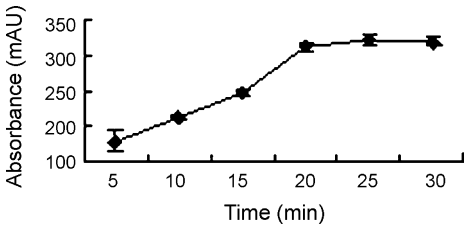

Fig. 4. Effect of incubation time on the extraction efficiency. Experimental condition: 5% Triton X-114, 45 °C, with addition of NaOH.

10 min produced low precision signal responses (relative standard deviation of 7.8%). As can be seen from Fig. 4, extraction time of 20 min was enough to give the highest signal response, so an incubation time of 20 min was recommended. In fact, the recovery values did not increase significantly from 10 to 20 min extraction processes. The improvement on signal response was achieved by the smaller phase volume ratio caused by longer equilibration time. This phenomenon was in quite accordance with a previous report [18], and was only significant within the time range from 5 to 20 min during this experiment. The procedure was then accelerated by centrifugation at 3500 rpm ($890 \times g$) for 5 min, which was enough to get a complete phase separation.

3.3. Calibration and validation

The spiked standard samples at six concentration levels over the range 0.08–20 μg/ml were prepared and analyzed in three consecutive analytical runs, two groups for each run. Standard curves were constructed using weighted ($1/x^2$) linear least-squares regression analysis of the observed peak area ratios of arbidol and the IS against concentration. The mean regression equation for the calibration curve was $y = 5 \times 10^{-2} x + 9.7 \times 10^{-4}$ with a correlation coefficient above 0.998. The LLOQ of this method was 80 ng/ml for arbidol in rat plasma (S/N = 10).

QC samples at three concentrations (0.2, 2 and 16 μg/ml) were analyzed to assess the precision and accuracy of the proposed method. Six replicates were analyzed in each of three consecutive analytical runs. The accuracy was expressed by the relative error (RE), and the precision was evaluated by the relative standard deviation (RSD). Recovery of extraction procedure was also evaluated at three concentration levels. It was determined by comparing the mean peak areas ($n = 6$ for each concentration level) obtained from plasma samples spiked before extraction with those from plasma samples spiked after extraction. The results are listed in Table 1, which indicate good analytical characteristics of this assay.

Table 1
Precision, accuracy and recovery of arbidol assay in rat plasma ($n = 6$)

Concentration (ug/ml)	Precision (RSD %)		Accuracy (RE %)	Recovery (%)
	Intra-day	Inter-day		
0.2	6.6	4.9	−1.6	89.7 ± 3.6
2	4.4	8.8	−5.0	91.4 ± 3.3
16	2.8	2.1	3.4	92.7 ± 0.98

To evaluate sample stability after freeze–thaw cycles and at room temperature, six replicates of QC samples at each of 0.2, 2 and 16 μg/ml concentrations were subjected to three freeze–thaw (−20 to 25 °C) cycles or were stored at room temperature for 4 h before sample processing, respectively. Six replicates of QC samples at each of 0.2, 2 and 16 μg/ml concentrations were processed and stored under autosampler condition for 12 h. Long-term cold storage stability was also evaluated at three concentration levels. 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 concluded stable if bias of them were within ±15% of the actual value [19]. Table 2 summarizes the stability data of the freeze and thaw, short-term, post-preparative as well as long-term test of arbidol, which indicate good stability of arbidol under these conditions.

3.4. Pharmacokinetic study of arbidol in rats

The plasma concentrations of arbidol at different points were expressed as mean ± SD, and the mean concentration–time curves were plotted (Fig. 5). All the pharmacokinetic data listed

Table 2
Summary of stability of arbidol in rat plasma ($n = 6$)

Concentration found (ng/ml) (mean ± SD)	Concentration added (μg/ml)		
	0.2	2	16
Freeze and thaw stability			
At the beginning	0.200 ± 0.013	1.933 ± 0.029	16.167 ± 0.590
After three freeze–thaw cycles	0.194 ± 0.012	1.780 ± 0.013	17.038 ± 0.243
Bias (R.E. %)	−3.1	−6.8	5.4
Short-term room temperature stability			
At the beginning	0.201 ± 0.025	1.818 ± 0.160	16.624 ± 0.257
After 4 h at room temperature	0.189 ± 0.009	1.901 ± 0.118	15.892 ± 0.506
Bias (R.E. %)	−6.4	4.6	−4.4
Post-preparative stability			
Immediately after extraction	0.203 ± 0.012	1.974 ± 0.144	16.539 ± 0.457
After 12 h in auto sampler condition (4 °C)	0.191 ± 0.022	1.932 ± 0.321	15.892 ± 0.506
Bias (R.E. %)	−5.8	−2.1	−3.9
Long-term cold storage stability			
At the beginning	0.203 ± 0.012	2.019 ± 0.111	15.810 ± 0.164
After 15 days at −20 °C	0.198 ± 0.007	1.823 ± 0.078	16.765 ± 0.271
Bias (R.E. %)	−2.3	−9.6	6.0

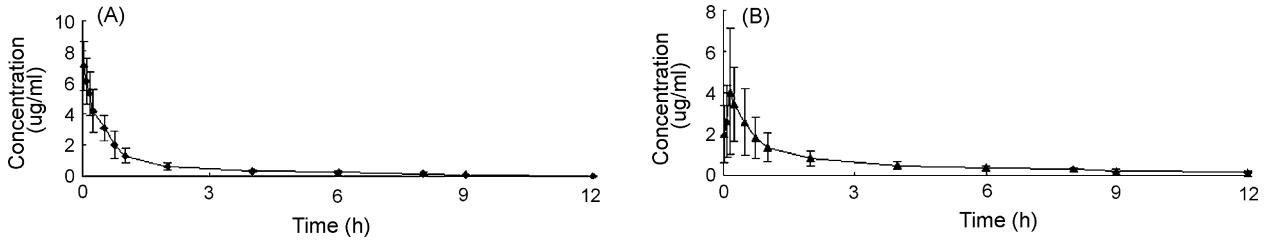

Fig. 5. Mean plasma concentration–time curve for arbidol in rat plasma after intravenous (A) and oral (B) administration (each point and bar represents the mean ± SD, $n = 6$).

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

Parameter	Estimate (mean ± SD)	
	Intravenous	Oral
t_{max} (h)	0.0333 ± 0	0.180 ± 0.063
C_{max} (μg/ml)	7.04 ± 1.59	4.71 ± 2.36
$t_{1/2}$ (h)	3.05 ± 1.28	3.28 ± 0.70
AUC_{0-t} (μg h/ml)	6.12 ± 1.51	6.79 ± 2.75
$AUC_{0-\infty}$ (μg h/ml)	6.70 ± 1.77	7.56 ± 2.88

in Table 3 were obtained using the DAS 2.0 statistical software (Pharmacology Institute of China). Compared with intravenous administration, pharmacokinetic study of arbidol in rats after oral administration of the same dosage (9 mg/kg) was also attempted. But the whole plasma concentration–time profile was unavailable since the concentrations after absorption were not high enough to be determined under the experimental conditions. When the dosage became higher (54 mg/kg), a complete plasma concentration time curve was obtained. Arbidol with an acceptable bioavailability of 18.8% was absorbed quickly into the systemic circulation and reached its maximum concentration within 10 min after oral dosing. The courses of arbidol in rats after intravenous (9 mg/kg) and oral (54 mg/kg) administration possessed an identical elimination procedure with $t_{1/2}$ parameters of 3.05 and 3.28 h, respectively. The results indicated that arbidol in oral preparations with appropriate dose might act no less quickly and effectively than the intravenous ones, which would be beneficial to reducing the production cost.

4. Conclusion

The cloud point extraction technique was applied as an effective method for the extraction and preconcentration of arbidol from rat plasma samples. Coupled with HPLC–UV detector, the method has been proved to be simple, rapid and reliable for arbidol assay in biological samples.

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