Available online at www.sciencedirect.com
ScienceDirect
ELSEVIER
Journal of Pharmaceutical and Biomedical Analysis xxx (2006) xxx–xxx
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

Determination of arbidol in human plasma by LC–ESI-MS

Xiao Liu, Yu-Wei Huang, Juan Li, Xiao-Bo Li, Kai-Shun Bi, Xiao-Hui Chen *

Department of Pharmaceutical Analysis, Shenyang Pharmaceutical University, Wenhua Road 103, Shenyang 110016, China

Received 8 May 2006; received in revised form 23 June 2006; accepted 26 June 2006

Abstract

A sensitive, specific and accurate method for determination of arbidol in human plasma was developed. Arbidol and internal standard were extracted from plasma samples by liquid–liquid extraction with diethyl ether. The chromatographic separation was accomplished on a Shiseido C_{18} 3 μm analytical column (100 mm × 2.0 mm i.d.) at a flow rate of 0.3 mL/min isocratically. Detection was performed on a single quadrupole mass spectrometer by selected ion monitoring (SIM) mode via electrospray ionization (ESI) source. The method had a chromatographic run time of 6 min and a good linear relationship over the range 1–1000 ng/mL. The limit of quantitation for arbidol in plasma was 1 ng/mL. The intra-day and inter-day precision (R.S.D.%) was lower than 7% and accuracy ranged from 95 to 105%. The proposed method enables unambiguous identification and quantification of arbidol in vivo and has been successfully applied to study the pharmacokinetics of arbidol in healthy male Chinese volunteers.

Keywords: Arbidol; LC–MS; Human plasma; 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. The compound can activate phagocytic activity of macrophages, and stimulate some forms of cellular and humoral immunity [1]. Metabolic and pharmacokinetics studies in animals have shown that arbidol is absorbed and distributed quickly into tissues and organs after oral administration [2]. 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 [3,4]. Potentially large number of samples in clinical studies needs a rapid and reliable assay. A literature survey revealed very few reports on the analysis of arbidol [5,6]. For in vivo, only Rainer Metz described a method using HPLC-UV to determine the concentration of arbidol in human plasma with a LLOQ of 5 ng/mL [6]. But its adoption of ion-pair chromatographic condition results in poor reproducibility.

Liquid chromatography coupled with mass spectrometry (LC–MS) is an analytical tool, which is widely used for concentration determination of drugs in biological fluids. It ensures low-level detection and quantitation of drugs with high degree of specificity at relatively short time of analysis without a need for complete chromatographic resolution of analytes. With basic side chains, arbidol demonstrates good mass spectrometric performance. So we developed a simple and accurate method for the quantification of arbidol in human plasma using liquid chromatography–mass spectrometry technique with a LLOQ of 1 ng/mL. Prior to the application of the method to bio-study, its specificity, sensitivity, accuracy, reproducibility and reliability were confirmed by a rigorous pre-study validation of the analytical method in accordance with international guidelines [7].

2. Experimental

2.1. Materials 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%) were kindly provided by Professor Ping Gong from Shenyang Pharmaceutical University. The deionized water was purified by Milli-Q water system (Millipore, Bedford, MA, USA). Methanol (Waters, USA) was of HPLC grade. Formic acid, sodium carbonate and diethyl ether, which were all of analytical grade were commercially available. Blank plasma was obtained

* Corresponding author. Tel.: +86 24 23986259; fax: +86 24 23986259.
E-mail address: cxh_syphu@yahoo.com.cn (X.-H. Chen).

0731-7085/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2006.06.039

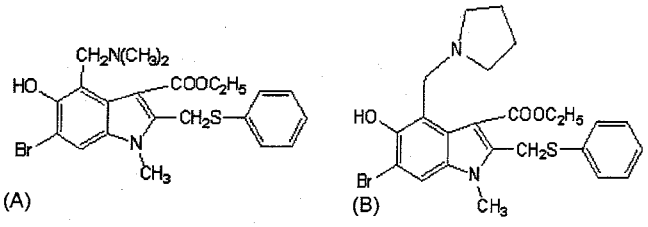

Fig. 1. Chemical structures of arbidol (A) and internal standard (B).

from the Blood Supply Center (Shenyang, China) and was stored in a freezer at −20 °C until needed.

2.2. Instruments and operating conditions

The assay was performed using Shimadzu (Japan) LC–MS 2010A system. Liquid chromatographic separation was achieved on a Shiseido 3 μm C_{18} column (100 mm × 2.0 mm i.d., Japan), which was preceded by a guard column (C_{18}, 30 mm × 2.0 mm, Phenomenex, Torrance, CA, USA). The column and autosampler tray temperature were kept constant at 30 and 14 °C, respectively. The mobile phase was a methanol–water–formic acid mixture (72:28:0.1, v/v/v). The flow rate was 0.3 mL/min.

The ESI source was set at positive ionization mode. The $[M+H]^+$, *m/z* 479.0 for arbidol and $[M+H]^+$, *m/z* 504.9 for IS were selected as detecting ions, respectively. The MS operating conditions were optimized as follows: drying gas, 1.5 L/min; CDL temperature, 250 °C; block temperature, 200 °C; detector voltage, 1.5 kV.

2.3. Preparation of standard solutions, calibration standards and quality control samples (QC)

Both of the stock solutions of arbidol and IS were prepared in methanol at concentration levels of 2.5 and 1 mg/mL, respectively. Standard solutions were prepared by diluting the stock solutions above with methanol. The concentration of working solution for internal standard was 10 μg/mL. All arbidol and IS solutions were stored at 4 °C, protected from light.

Calibration standards of arbidol at concentrations of 1, 2.5, 10, 50, 200, 1000 ng/mL were prepared by spiking appropriate amount of the standard solutions in blank plasma. Quality control (QC) samples (of low, medium and high concentration) at 2.5, 50 and 800 ng/mL were prepared in the same way as the calibration standards and stored at −20 °C.

2.4. Sample preparation

Plasma samples (500 μL) were spiked with 50 μL of internal standard (10 μg/mL), 50 μL of methanol and 300 μL of 0.04 M Na_2CO_3, and extracted for 5 min with 3 mL diethyl ether. After centrifugation (2000 × *g*, 4 °C, 5 min), the organic phase was transferred to another vial and evaporated to dryness in a thermostatically controlled water-bath at 40 °C under a slight stream of nitrogen. Then the residue was dissolved in 100 μL methanol, 20 μL of it was used for LC–MS analysis. According to Rainer Metz' research, arbidol solution was sensitive to sunlight [6]. So during the extraction procedure, samples were kept protected from sunlight.

2.5. Method validation

2.5.1. Specificity

The specificity of the assay for the analytes versus endogenous substances in the matrix was tested by comparing the lowest concentration in the calibration curve standards with reconstitutions prepared with drug-free plasma from six volunteers.

2.5.2. Linearity and LLOQ

Calibration standards of six concentrations for arbidol (1, 2.5, 10, 50, 200, 1000 ng/mL) were extracted and analyzed. Peak-area ratios of arbidol to IS obtained from SIM chromatograms were utilized for construction of calibration curves, using weighted liner least square regression (weighting factor was 1/*C*) of the plasma concentrations and the measured ratios. The linearity of the calibration curve was conformed by plotting the peak-area ratios versus the concentrations of arbidol. The low limit of quantification for arbidol was estimated in accordance to the base line noise, considering a signal-to-noise ratio of 10:1. Calibration curves were prepared and assayed along with each batch of clinical plasma samples.

2.5.3. Precision and accuracy

The precision and accuracy of the assay were determined at low, medium and high concentrations of arbidol by replicate analyses of QC samples. Intra-day precision was determined by repeated analyses of each QC sample on 1 day ($n=5$). Inter-day precision was determined by repeated analyses on 5 consecutive days ($n=1$ series per day). The concentration of each sample was determined using a calibration curve prepared each day. Accuracy was defined as the relative deviation in the calculated value (*E*) of a standard from that of its true value (*T*), expressed as a percentage (RE%). It was calculated using the formula:

$$\mathrm{RE\%} = \frac{E-T}{T} \times 100 \quad (1)$$

Precision was defined as the relative standard deviation (S.D.) from the mean (*M*), calculated using the equation:

$$\mathrm{R.S.D.\%} = \frac{\mathrm{S.D.}}{M} \times 100 \quad (2)$$

2.5.4. Extraction recovery and ionization

The extraction efficiency of arbidol from human plasma matrix was evaluated by comparing the mean detector responses of six processed QC samples of low, medium and high concentrations to the mean detector responses for six standard solutions of equivalent concentration. Similarly, the recovery of internal standard was evaluated by comparing the mean detector responses of six processed plasma samples to the mean detector responses of six standard solutions of identical concentration.

Ion suppression of ionization was evaluated by comparing the absolute peak area of control plasma extracted and then spiked

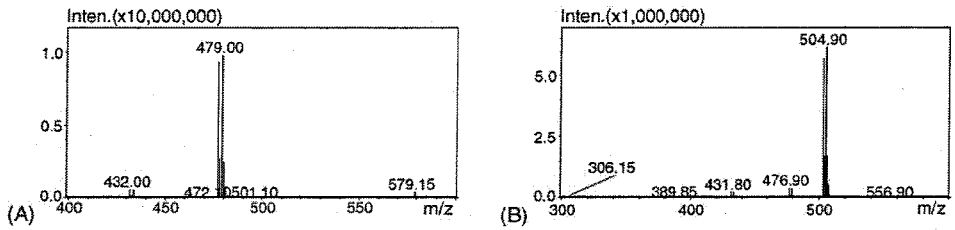

Fig. 2. Positive scan mass spectra of arbidol (A) and IS (B) at 1.5 kV detector voltage.

with a known amount of drug, to that of neat standard injected in the same reconstitution solvent.

2.5.5. Stability

To evaluate sample stability after three freeze–thaw cycles and at room temperature, six replicates of QC samples at each of 2.5, 50 and 800 ng/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 2.5, 50 and 800 ng/mL concentrations were processed and stored under autosampler condition for 24 h. Long-term cold storage stability was also evaluated at three concentrations. Stability was assessed by comparing the mean concentration of the stored QC samples with the mean concentration of freshly prepared QC samples. Stability samples were to be concluded stable if bias of them were within ±15% of the actual value [7].

2.5.6. Pharmacokinetic study

The method developed was used to investigate pharmacokinetic parameters of arbidol in 20 healthy male Chinese volunteers. Each of the volunteers received 200 mg arbidol of dispersible tablet formulation. Venous blood samples were collected at different time points (0, 0.125, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 7, 12, 24, 48, 72 h) after a single oral dose. They were withdrawn into heparinised tubes, and centrifuged at 1000 × *g* for 5 min at 4 °C. The plasma samples obtained were then stored at −20 °C until analysis, protected from light. The study had been approved by the local ethics committee. All subjects gave their written informed consent before participation in the study.

3. Results and discussion

3.1. Mass spectra analysis

The full scan mass spectra of arbidol and IS after direct injection in mobile phase are presented in Fig. 2. The mass spectrometric parameters were optimized to obtain the higher signal for the selected ion *m/z* 479.0 of arbidol $[M+H]^+$. With an addition of formic acid to the mobile phase, the sensitivity and the shape of target peaks were both improved. What it must be explained here is that, because the structure of arbidol consists of a bromine atom, the signal peak was actually one of the isotopes. The same was true of the internal standard.

3.2. Method validation

3.2.1. Specificity

No endogenous interference was observed at retention times of arbidol (4.3 min) and internal standard (4.5 min) because of the high selectivity of the SIM mode. Representative chromatograms of blank plasma, spiked plasma sample and subject sample are shown in Fig. 3.

3.2.2. Linearity and LLOQ

The calibration curves, which related the concentrations of arbidol to the area ratios of arbidol to IS, showed good linearity in the range of 1–1000 ng/mL. The typical calibration curve for arbidol had a slope of 0.010484 ± 0.000487, an intercept of 0.000155 ± 0.000075 and $r = 0.9998$ ($n = 5$). The LLOQ for arbidol in plasma was 1 ng/mL (lowest standard level) with coefficient of variation of 4.66% and accuracy of 100.41% ($n = 6$). The data above show that this assay is sensitive enough for pharmacokinetics study of arbidol in vivo.

Both uniform and 1/*x* weighting gave linear regression analysis with $r > 0.999$, however, the use of 1/*x* weighting for the standard curve resulted in preferable prediction of standard samples across the concentration range. Most notably, with a minor intercept, the 1/*x* weighting yielded better accuracy at lower standard levels.

3.2.3. Precision and accuracy

Intra-day and inter-day precision and accuracy were determined by measuring QC samples at three concentrations as described in Section 2. The intra-day precision (R.S.D.%) was found to be <6% ($n = 5$) and RE% ranged from −3.20 to 0.13%. The inter-day precision (R.S.D.%) was found to be <7% ($n = 5$) and RE% ranged from −4.80 to 0.48% (Table 1).

3.2.4. Extraction recovery and ionization

The mean extraction recoveries of the method for arbidol determined using six replicates of QC samples at three con-

Table 1
Precision and accuracy of the assay for determination of arbidol in plasma ($n = 5$)

Concentration added (ng/mL)	Intra-day			Inter-day		
	2.5	50.0	800.0	2.5	50.0	800.0
Mean concentration found (ng/mL)	2.42	49.32	801.05	2.38	47.96	803.88
RE (%)	−3.20	−1.36	0.13	−4.80	−4.08	0.48
R.S.D. (%)	4.50	5.18	4.81	5.15	6.44	5.24

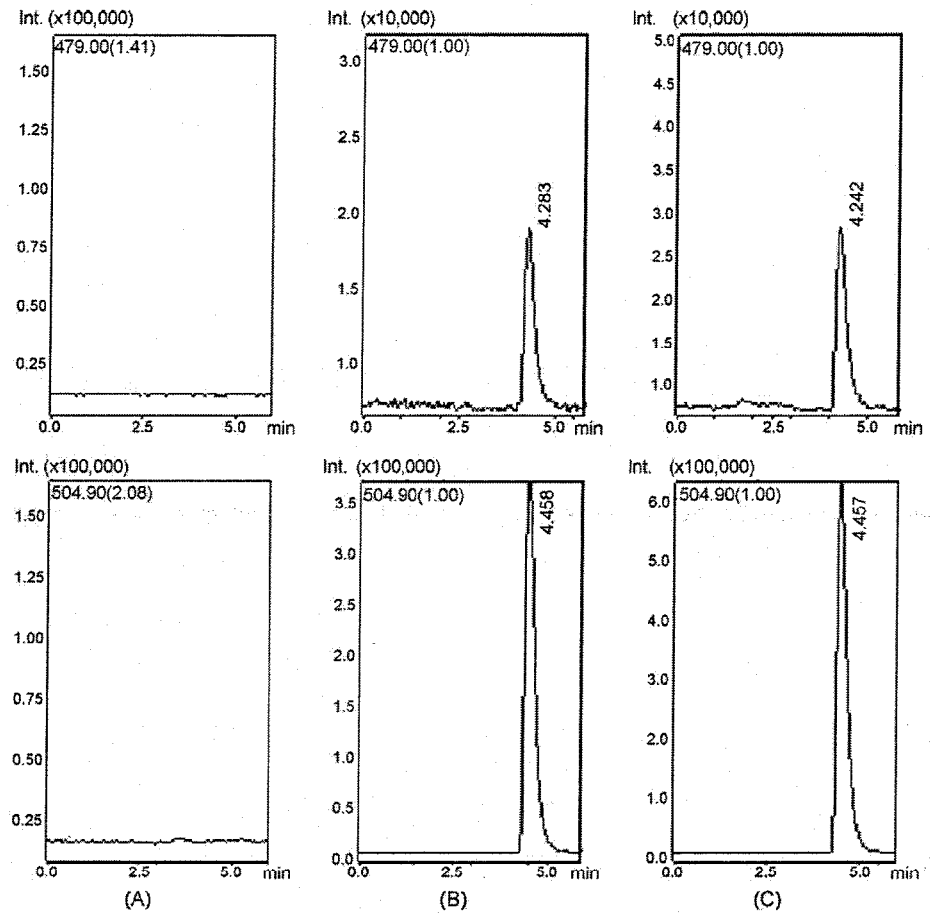

Fig. 3. Typical SIM chromatograms of blank plasma (A), blank plasma spiked with arbidol (1 ng/mL, LLOQ) and IS (B), and subject plasma sample obtained from a volunteer 48 h after administration (C).

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

Concentration found (ng/mL) (mean ± S.D.)	Concentration added (ng/mL)		
	2.5	50.0	800.0
Freeze and thaw stability			
At the beginning	2.43 ± 0.11	49.21 ± 2.23	808.90 ± 44.82
After three freeze–thaw cycles	2.23 ± 0.24	44.89 ± 3.45	827.79 ± 52.33
Bias (%)	−8.23	−8.78	2.33
Short-term room temperature stability			
At the beginning	2.58 ± 0.14	48.32 ± 2.44	791.07 ± 40.88
After 4 h at room temperature	2.40 ± 0.21	49.50 ± 5.01	803.03 ± 45.65
Bias (%)	−6.98	2.44	1.51
Post-preparative stability			
Immediately after extraction	2.54 ± 0.19	48.30 ± 2.67	789.24 ± 39.98
After 24 h in auto sampler condition	2.62 ± 0.23	46.45 ± 4.03	770.12 ± 43.01
Bias (%)	3.15	−3.83	−2.42
Long-term cold storage stability			
At the beginning	2.26 ± 0.16	46.85 ± 2.89	754.03 ± 43.41
After 45 days at −20 °C	2.12 ± 0.29	43.52 ± 4.54	723.43 ± 50.17
Bias (%)	−6.19	−7.11	−4.06

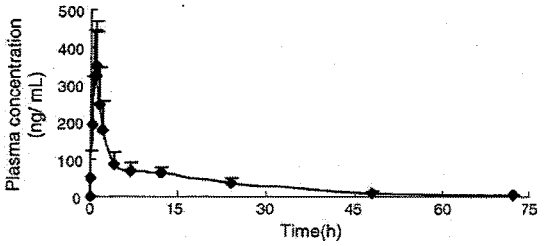

Fig. 4. Mean plasma concentration of arbidol–time profile in 20 healthy volunteers after a 200 mg oral dose.

centration levels (2.5, 50, 800 ng/mL) were found to be 86.3% (R.S.D., 4.23%), 89.8% (R.S.D., 4.14%) and 88.3% (R.S.D., 3.37%), respectively. The recovery for internal standard was 90.3% (R.S.D., 2.96%).

As for ionization, the peak area ratios of arbidol and IS after spiking evaporated plasma samples at three concentration levels (2.5, 50, 800 ng/mL) compared to neat standard solutions ranged from 98 to 106%, indicating that the evaluated method was free from matrix effect.

3.2.5. Stability

Stability of arbidol during sample handing (freeze–thaw and short-term room temperature), long-term cold storage (−20 °C, 45 days), and the stability of processed samples were evaluated (Table 2). Arbidol was stable for at least 4 h at room temperature in plasma samples, for 24 h in autosampler condition after preparation, for 45 days under cold storage and within three freeze–thaw cycles in plasma samples.

3.2.6. Application

The validated method was used to assay arbidol in human plasma samples obtained from 20 healthy Chinese volunteers in a pharmacokinetic study. Calibration levels were identical to those that used during validation procedures. The values obtained from the calibration levels and the resulting calibration graphs were in a similar range as observed during validation. The coefficient of correlation (r) was above 0.998 in each batch. Precision and accuracy for each batch at three levels of QC samples were all below 15%. The concentration of arbidol reached a maximum (C_{max}) of 418.5 ± 85.2 ng/mL, approximately 40 min (0.7 ± 0.3 h, T_{max}) after administration. The plasma elimination half-life ($t_{1/2}$) was determined as 7.5 ± 5.1 h. The value of area under the plasma concentration–time curve from 0 to the last measured concentration time (AUC_{0-t}) was calculated as 2011.8 ± 538.1 ng h/mL and it constituted 88% of the AUC value extrapolated from 0 to infinity (2285.9 ± 645.4 ng h/mL), which indicated a suitability of the analytical method for pharmacokinetic investigations in humans. The mean plasma concentration–time curve of 20 healthy volunteers after a single oral dose of 200 mg arbidol of dispersible tablet formulation is shown in Fig. 4.

4. Conclusion

A selective, sensitive and accurate LC–MS analytical method for the determination of arbidol in human plasma has been successfully developed and validated. The method has advantages of short analysis time, non-tedious sample preparation and the avoidance of complete separation of the analytes from plasma matrix. Therefore, the method proposed could be of great use for pharmacokinetics, bioavailability or bioequivalence studies of arbidol in biological samples.

Acknowledgement

The authors would like to thank Prof. Ping Gong (Shenyang Pharmaceutical University, China) for supplying arbidol and the internal standard.

References

[1] H. Cai, Y. Zhao, C. Zhao, P. Gong, Bioorg. Med. Chem. 14 (2006) 911–917.
[2] R.G. Glushkov, Drugs Future 17 (1992) 1079–1081.
[3] A.M. Shuster, V.I. Shulimov, V.A. Shevtsov, G.G. Marin, V.N. Kozlov, Voen. Med. Zh. 325 (2004) 44–45.
[4] M.Z. Wang, B.Q. Cai, L.Y. Li, J.T. Lin, N. Su, H.X. Yu, H. Gao, J.Z. Zhao, L. Liu, Zhongguo Yi Xue Ke Xue Yuan Xue Bao 26 (2004) 289–293.
[5] L. Miller, R. Bergeron, J. Chromatogr. A 658 (1994) 489–496.
[6] R. Metz, P. Muth, M. Ferger, V.G. Kukes, H. Vergin, J. Chromatogr. A 810 (1998) 63–69.
[7] Guidance for industry, Bioanalytical method validation (updated 2001). http://www.fda.gov/cder/guidance/index.htm.