Pharmacokinetics, Metabolism, and Excretion of the Antiviral Drug
Arbidol in Humans

Pan Deng, Dafang Zhong, Kate Yu, Yifan Zhang, Ting Wang, and Xiaoyan Chen∗

Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China; Waters Corporation, MA, USA; and First Affiliated Hospital of Lanzhou University, Lanzhou, China

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* Corresponding author: Xiaoyan Chen

Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 501 Haike Road, Shanghai 201203, China
Phone/Fax: 86-021-50800738
Email: xychen@mail.shcnc.ac.cn

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ABSTRACT

Arbidol is a broad-spectrum antiviral drug, which is clinically used to treat influenza. In this study, the pharmacokinetics, metabolism, and excretion of arbidol were investigated in healthy male Chinese volunteers after a single oral administration of 200 mg of arbidol hydrochloride. A total of 33 arbidol metabolites were identified in human plasma, urine, and feces. The principal biotransformation pathways included sulfoxidation, dimethylamine N-demethylation, glucuronidation, and sulfate conjugation. The major drug-related component in the plasma was sulfinylarbidol (M6-1), followed by unmetabolized arbidol, N-demethylsulfinylarbidol (M5), and sulfonylarbidol (M8). The exposures of M5, M6-1, and M8, as determined by the metabolite-to-parent AUC_0-t ratio, were 0.9 ± 0.3, 11.5 ± 3.6, and 0.5 ± 0.2, respectively. In human urine, glucuronide and sulfate conjugates were detected as the major metabolites, accounting for 6.3% of the dose excreted within 0 to 96 h after drug administration. The fecal specimens mainly contained the unchanged arbidol, accounting for 32.4% of the dose. Microsomal incubation experiments demonstrated that liver and intestines were the major organs that metabolize arbidol in humans. CYP3A4 was the major isoform involved in arbidol metabolism, whereas the other P450s and FMOs played minor roles. These results indicated possible drug interactions between arbidol and CYP3A4 inhibitors and inducers. Further investigations are needed to understand the importance of M6-1 in the efficacy and safety of arbidol, because of its high plasma exposure and long elimination half-life (25.0 h).
Arbidol (ethyl-6-bromo-4-[(dimethylamino)methyl]-5-hydroxy-1-methyl-2-[(phenylthio)methyl]-indole-3-carboxylate) is a broad-spectrum antiviral compound. It was first marketed in 1993 for prophylaxis and treatment of influenza A and B infection (1). Recently, Teissier et al. reported that arbidol could inhibit the viral glycoprotein conformational changes during the membrane fusion by interacting with the phospholipid membrane and protein motifs enriched in aromatic residues (2). Clinical trials indicated that 200 mg of arbidol taken three times daily for 5 days to 10 days reduces the duration of influenza by 1.7 to 2.65 days (3). Recent studies have extended the inhibitory activity of arbidol to other human viruses, such as hepatitis B and C viruses, the rhinovirus 14, bird viruses, chikungunya virus, and respiratory syncytial virus (4, 5).

Circulating metabolites have been known to contribute to or alter the pharmacological activities of the parent drug. The safety of circulating metabolites should be considered. Furthermore, identifying drug metabolic pathways is also important for predicting drug-drug interactions (DDIs). However, the current understanding of arbidol metabolism in humans is incomplete, and only a single study has been reported on the human urinary metabolites identification after oral drug administration. Glucuronide arbidol and glucuronide sulfinylarbidol were detected as the major metabolites in human urine (6). The limited pharmacokinetics of arbidol in healthy human volunteers has been described, which showed its rapid absorption ($T_{max}$ 1.6 – 1.8 h) and slow elimination ($t_{1/2}$ 16 – 21 h) (1, 7, 8). No circulating arbidol metabolites have been detected or characterized thus far.

The objectives of this study are to investigate the metabolic profile, the routes of excretion, and the pharmacokinetics of arbidol in healthy male volunteers after a single oral dose of 200 mg of arbidol hydrochloride. The metabolites in circulation and excreta were identified using ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC/Q-TOF MS), and the major metabolites were synthesized for structure confirmation. The pharmacokinetic profiles of arbidol and its primary metabolites were characterized. The enzymes responsible for the principal metabolic pathways were identified.
using recombinant P450s and FMOs, human liver microsomes (HLMs), human intestine microsomes (HIMs), and human kidney microsomes (HKMs) with and without P450 inhibitors and heat inactivation of FMOs.

Materials and Methods

Materials. Arbidol hydrochloride capsules (100 mg/capsule, equivalent to 89 mg base/capsule) were purchased from Shijiazhuang No. 4 Pharmaceutical Co., Ltd (Hebei, China). Arbidol hydrochloride was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Arbidol derivatives including oxidative S-dealkylated arbidol, N-demethylsulfinylarbidol, sulfinylarbidol, 4′-hydroxylated arbidol, N-demethylsulfonylarbidol, and sulfonylarbidol were synthesized by Hebei University of Science and Technology (Hebei, China) and served as synthetic standards to confirm the metabolites structures. These compounds were >99% pure. Pooled HLMs and HKMs, recombinant P450 enzymes (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5, and CYP4A11), and FMO isoforms (FMO1, FMO3, and FMO5) were purchased from BD Gentest (Woburn, MA, USA). Pooled HIMs were purchased from Xenotech LLC (Lenexa, KS, USA). The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA): β-glucuronidase from Helix pomatia (Type H-1), 1-aminobenzotriazole (1-ABT), benzydamine, NADPH, α-naphthoflavone, ticlopidine, quinidine, clomethiazole, ketoconazole, and all solvents used for liquid chromatography/mass spectrometry (LC/MS) analysis. Purified water was generated using a Milli-Q Gradient system (Molsheim, France).

Study Design, Dosing, and Sample Collection. This was an open-label, nonrandomized, single-dose study. Four male subjects with a mean age of 24 years (ranged from 22 to 26 years) in good physical health, as shown by medical examination and history, vital signs, 12-lead electrocardiogram, and laboratory tests, were recruited. Body mass index range was 21–23 kg/m². The study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Lanzhou University (Lanzhou, China). Written informed consents were obtained from all subjects before enrollment. The subjects received a single oral dose of
200-mg arbidol hydrochloride capsules. Blood samples (4.5 ml) were collected into heparinized tubes predose and at 0.125, 0.25, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 12, 24, 36, 48, and 72 h postdose. Plasma was harvested by centrifugation and stored at −20°C until analysis. Urine samples were collected predose and at 0 to 12 h, 12 to 24 h, 24 to 48 h, 48 to 72 h, and 72 to 96 h postdose. Fecal samples were collected predose and thereafter up to 96 h postdose. Each portion was diluted with five volumes of methanol and homogenized. The urine and homogenized feces were stored at −20°C until analysis. Subjects were provided with standard meals at approximately 4 and 10 h after drug dosing.

**Metabolite Profiling. Sample Preparation and β-glucuronidase Hydrolysis.** Representative pooled samples were prepared for metabolite profiling experiments. The plasma samples were segregated by sampling time, and equal volumes of plasma samples from all subjects were pooled. The urine samples and fecal homogenate from all subjects were pooled by combining volumes proportional to the total volume or weight excreted by each subject for each collection interval. To a 50 μl aliquot of pooled plasma, urine, and fecal homogenate samples were added 200 μl of methanol. After vortex-mixed and centrifuged at 11,000 g for 5 min, the supernatant was transferred into a glass tube, evaporated to dryness under a stream of nitrogen at 40°C and then reconstituted in 100 μl of methanol and 5 mM ammonium acetate (1:1, v/v). A 10 μl aliquot of the reconstituted solution was injected onto UPLC/Q-TOF MS for analysis. For enzymatic incubation, a 50 μl aliquot of the urine sample was mixed with 50 μl of β-glucuronidase (in 1 M citrate buffer solution at pH 5.0). The mixture was incubated at 37°C for 16 h. The effect of the glucuronidase was studied by comparing the LC/MS peak intensities for compounds of interest before and after enzymatic incubation. The compounds of interest included glucuronide conjugates and their hydrolyzed forms.

**UPLC/Q-TOF MS Analysis.** Chromatographic separation for metabolite profiling was achieved using an Acquity UPLC system (Waters Corp., Milford, MA, USA) on an Acquity UPLC BEH column (1.7 μm, 2.1 mm × 50 mm, Waters Corp.). The mobile phase was a mixture of (A) 0.05% formic acid in 5 mM ammonium acetate and (B) methanol. The gradient elution started from 10% B, maintained for 1 min, increased linearly to 57% B over...
24 min, and then increased linearly to 100% B over the next 2 min, and finally decreased to
10% B to re-equilibrate the column. The column temperature was set at 35°C and the flow
rate was 0.4 ml/min. The eluent was monitored by UV detection at 316 nm. The MS detection
was conducted using a Synapt Q-TOF high-resolution mass spectrometer (Waters Corp.,
Milford, MA, USA) operated in positive ion electrospray (ES +ve) mode. The mass range of
m/z 80–1000 was acquired. Nitrogen and argon were employed as the desolvation gas and
collision gas, respectively. Desolvation temperature was set at 350°C and source temperature
was at 100°C. Leucine enkephalin was used as a lock mass compound ([M + H]⁺ m/z
556.2771) for accurate mass measurements, and was infused into the LockSpray ion source
via a separate ionization probe. Data acquisition was performed using the MS² scan function,
which was programmed with two independent collision energies (CE). At low collision energy,
the transfer CE and trap CE were 2 eV and 3 eV, respectively. At high collision energy, the
transfer CE and trap CE were 4 eV and ramped from 15 eV to 30 eV, respectively. In this
manner, the precursor ions and fragmentation information were obtained in a single run. The
mass spectrometer was operated using MassLynx 4.1 software. The metabolites were mined
from the data using the mass defect filtering function (40 mDa tolerance window) with the
MetaboLynx XS subroutine of the MassLynx software.

**Quantification of Arbidol and its Metabolites in Human Plasma.** The concentrations of
arbidol, M5, M6-1, and M8 in plasma were determined using a validated liquid
chromatography–tandem mass spectrometry (LC–MS/MS) method. Debutyldronedarone was
used as the internal standard (IS). A 50 µl aliquot of plasma containing the IS was treated with
methanol (200 µl) and centrifuged. The supernatant was diluted with an equal volume of the
mobile phase, and 5-µl aliquots were injected onto the LC–MS/MS system. Chromatographic
separation was achieved on a Gemini C18 column (50 mm × 2.0 mm, 5 µm) using methanol:
5 mM ammonium acetate containing 0.1% formic acid as the mobile phase with gradient
eletion. Mass detection was carried out on an API 4000 mass spectrometer (Applied
Biosystems, Concord, Ontario, Canada) using an ES +ve mode. The multiple reaction
monitoring transitions selected were m/z 479 ([M + H + 2]⁺) to 281 for arbidol, m/z 481 ([M +
H + 2\] +) to 356 for N-demethylsulfinylarbidol, m/z 495 ([M + H + 2\] +) to 370 for sulfinylarbidol, m/z 511 ([M + H + 2\] +) to 466 for sulfonylarbidol, and m/z 501 ([M + H\] +) to 114 for the IS. The assay was linear over the concentration range of 2.00–2000 ng/ml for arbidol and sulfinylarbidol, and 0.50–500 ng/ml for N-demethylsulfinylarbidol and sulfonylarbidol.

**Pharmacokinetic Analysis.** The pharmacokinetic parameters were determined using the WinNonlin non-compartmental analysis computer program (version 5.3; Pharsight, Mountain View, CA, USA). The peak concentration (C_{max}) and the time to reach it (T_{max}) were determined directly from the experimental data. The terminal elimination phase rate constant (K_e) was estimated using the least-squares regression analysis of the plasma concentration-time data obtained during the terminal log-linear phase. The terminal phase half-life (t_{1/2}) was calculated as 0.693/K_e. The area under the plasma concentration-time curve (AUC) was calculated according to the linear trapezoidal method from 0 h to 96 h (AUC_{0-96}) or to infinity (AUC_{0-∞}). The oral clearance (CL/F) was calculated as dose/AUC_{0-∞}.

**Quantification of Arbidol and its Metabolites in Human Urine and Feces.** The concentrations of arbidol, M5, M6-1, and M8 in urine and feces were determined using the LC–MS/MS method. The urine and feces homogenates were prepared the same as the plasma, except the fecal extraction supernatant was diluted 100-fold with the mobile phase before analysis. The semi-quantification of conjugate metabolites in urine was performed with the UPLC-UV method. The gradient elution program was the same as that for metabolite identification, and the UV detector was set at 316 nm. The glucuronide conjugates (M18, M20-1, and M20-2) and sulfate conjugates (M10, M11-2, M14-1, and M15) were semi-quantified using arbidol as calibration standard. The percentage of dose excreted over 0 h to 96 h was then calculated as follows:

\[
\text{Excretion (\%) = \frac{\text{Moles of metabolites excreted in human urine or feces}}{\text{Moles of arbidol dosed to humans}}} \times 100
\]

**Incubation with HLM, HIM, HKM, and cDNA-Expressed P450s and FMOs.** Arbidol was incubated in triplicate with pooled HLM, HIM, HKM, human cDNA-expressed P450s (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6,
CYP2E1, CYP3A4, CYP3A5, and CYP4A11) or FMOs (FMO1, FMO3, and FMO5). The incubation mixtures (200 µl) contained potassium phosphate buffer (0.1 M, pH 7.4), individual P450 enzymes (50 pmol/ml), FMOs (4 pmol/ml), HLM (1 mg/ml), HIM (1 mg/ml), or HKM (1 mg/ml), arbidol (5.0 µM or 50 µM), and NADPH (2.0 mM). Arbidol stock solution was prepared in dimethyl sulfoxide (DMSO), and the final DMSO concentration in the incubation was 0.1% (v/v). The reactions were initiated with the addition of NADPH, and the incubations were performed at 37°C in a water bath. After 60 min of incubation, the reactions were terminated with an equal volume of ice-cold acetonitrile. Control samples without NADPH or substrate were included. The samples were analyzed using UPLC/Q-TOF MS.

For the microsomal stability assay, arbidol, M5, M6-1, and M8 (each at 5.0 µM) were individually incubated with HLMs in the presence of NADPH, using the same incubation conditions described above. The reactions were terminated at 0 (T₀), 5.0, 15, 30, and 60 min. The disappearance of test compound was monitored using UPLC/Q-TOF MS.

Inhibition of P450s or FMOs in HLMs and HIMs. The contribution of P450s and FMOs to arbidol metabolism was distinguished by selectively inhibiting each enzyme system. The chemical inhibitors used were ABT (1 mM) for all P450 enzymes, α-naphthoflavone (2 µM) for CYP1A2, ticlopidine (24 µM) for 2C19, quinidine (8 µM) for 2D6, clomethiazole (24 µM) for CYP2E1, and ketoconazole (2 µM) for CYP3A4/5. The incubation mixtures (200 µl, in triplicate) contained phosphate buffer (0.1 M, pH 7.4), HLM (1 mg/ml) or HIM (1 mg/ml), arbidol (5.0 µM or 50 µM), NADPH (2.0 mM), and a single P450 chemical inhibitor. The FMOs were inhibited by heating the HLM and HIM at 45°C for 5 min (without NADPH). The effect of heat inactivation on FMO activity was confirmed by the incubation of benzydamine (a selective FMO substrate) with preheated microsomes and determining the subsequent decrease in benzydamine N-oxide (m/z 326.187) production. The formation of metabolites was analyzed by UPLC/Q-TOF MS as described above. A comparison was made relative to the controls without inhibitor, and the enzyme activity was expressed as the percentage of the activity of the control.
Data Analyses. In the determination of the \textit{in vitro} \( t_{1/2} \) of arbidol, M5, M6-1, and M8, the peak areas were converted to the percentage of the compound that remained, using the \( T_0 \) peak area values as 100%. The ln percentage of the remaining test compound was plotted against incubation time, and the slope of the linear regression (\(-k\)) was used in the conversion to \textit{in vitro} \( t_{1/2} \). The intrinsic clearance (CL\textsubscript{int}, in ml/min/kg) was calculated using the following formula (9, 10):

\[
\text{CL}_{\text{int}} = \frac{0.693}{\ln 2} \frac{\text{ml incubation}}{\text{mg microsome}} \times \frac{48.8 \text{ mg microsome}}{25.7 \text{ gm liver}} \times \frac{25.7 \text{ gm liver}}{\text{kg b.w.}}
\]

To estimate the contributions of the different P450 and FMO isoforms to the formation of M5, M6-1, and M8 in human liver, the percentages of total normalized rate (\% TNR) were calculated as reported previously (11). This was done by multiplying the reaction rate of each isoform by the specific protein content in HLMs to yield the “normalized rate” (NR), which was expressed in pmol/min/mg microsomes. The NRs obtained were then summed up as the “total normalized rate” (TNR). The \% TNR for each isoform was then calculated according to the following equation:

\[
\% \text{TNR} = \frac{\frac{\text{NR}}{\text{TNR}} \times 100}{\frac{\text{pmol/min/pmol(P450 or FMO)} \times \text{pmol(P450 or FMO)/mg}}{\sum \left( \frac{\text{pmol/min/pmol(P450 or FMO)} \times \text{pmol(P450 or FMO)/mg}}{\text{pmol(P450 or FMO)/mg}} \right)}}
\]

Statistical analysis to investigate the effects of inhibitors was carried out in Microsoft Excel (version 2010) using a two-sided \( t \)-test for independent samples. All results are presented as mean \( \pm \) SD.

Results

Mass Spectral Fragmentation of Arbidol and Metabolite Standards. We previously reported the identification of arbidol metabolites using HPLC-ion trap mass spectrometry, and the MS fragmentation sequences of arbidol and its metabolites were proposed (6). In the present study, similar fragment pathways were observed for arbidol and the metabolite standards using Q-TOF MS. Typically, under the high collision energy, arbidol, \( N \)-demethylsulfonylarbidol, sulfonylarbidol, and 4′-hydroxylated arbidol produced abundant
fragments by sequential loss of dimethylamine (45.058 Da) or methylamine (31.039 Da),
acetaldehyde (44.026 Da), and the phenylthio radical (109.011 Da), phenylsulfonyl radical or
4′-hydroxyphenylthio radical (141.001 Da). For sulfinylarbidol and
N-demethylsulfinylarbidol, the major fragment ions were produced by a sequential loss of
phenylsulfanyl radical (125.006 Da), dimethylamine or methylamine, and acetaldehyde. The
high collision energy mass spectra and chromatographic behaviors of the detected metabolites
were compared with those of the parent compound and the available authentic standards to
characterize the structural modification.

**Metabolic Profiles in Human Plasma, Urine and Feces.** Table 1 lists the possible arbidol
metabolites, including their proposed elemental composition, chemical structures, retention
time of each chromatographic peak, and the characteristic mass spectral fragmentation ions.
The metabolic profiles of arbidol in plasma, urine and feces are shown in Fig. 1. The
identified metabolic pathways of arbidol in humans are shown in Fig. 2.

**Urine.** A total of 32 chromatographic peaks were observed in the urinary metabolic profile.
The peak at 23.0 min was assigned to unchanged arbidol because the retention time and mass
spectral fragmentation patterns were identical to those of arbidol. Likewise, the metabolite
peaks at 10.7, 14.9, 14.8, and 14.5 min (which contained two coeluted metabolites) were
identified as oxidative S-dealkylation metabolite (M1), N-demethylsulfinylarbidol (M5),
sulfinylarbidol (M6-1), N-demethylsulfonylarbidol (M7), and sulfonylarbidol (M8),
respectively. Another phase I metabolite eluted at 22.6 min was assigned as M3-2, and its
structure was proposed as dimethylamine N-demethylated arbidol. All the other metabolites
detected were phase II conjugates. The sulfate conjugation of M0, M1, M3-1, M3-2, M5,
M6-1, M7, and M8 produced metabolites M10, M4, M9-1, M9-2, M11-2, M14-1, M15, and
M16, respectively. Glucuronide conjugation of M0, M1, M3-1, M3-2, M5, M6, M7, and M8
yielded the metabolites M18, M13 (two isomers), M17-1, M17-2, M19 (two diastereomers),
M20 (two diastereomers and two isomers), M21, and M22, respectively. These glucuronide
conjugates were readily hydrolyzed by β-glucuronidase, and the chromatographic peaks for
the corresponding aglycones significantly increased after hydrolysis. These results supported
the structural assignment for glucuronide conjugate metabolites. The sulfate conjugates of
N-desmethyl M1 (M2), 1-methylindole N-desmethyl arbidol (M11-1), and
di-N-demethylsulfonylarbidol (M12), and the glucuronide conjugate of 4'-hydroxylarbidol
(M14-2) were detected at trace levels in the urine. Compared with the previously reported
results (6), the newly identified metabolic pathways included N-demethylation of
1-methylindole, 4'-hydroxylation, oxidative S-dealkylation, and di-N-demethylation. The
most abundant urinary metabolites were glucuronide arbidol (M18) and glucuronide
sulfinylarbidol (M20-1 and M20-2), which accounted for 1.5% and 2.1% of the dose,
respectively. The mean urinary excretion of sulfate arbidol (M10), sulfate
N-demethylsulfinylarbidol (M11-2), sulfate sulfinylarbidol (M14-1), and sulfate
N-demethylsulfonylarbidol (M15) amounted to 0.3%, 0.4%, 1.3%, and 0.7% of the dose. The
recovery of arbidol and its oxidative metabolites (M5, M6-1, and M8) from urine were less
than 1% of the dose.

Feces. A total of 24 metabolites, along with the parent drug, were detected in the extracts of
fecal homogenates. The product ion mass spectra and the UPLC retention times of these
metabolites were compared with those detected in human urine. The newly identified
metabolites were M3-1 and M6-2, which were eluted at 20.4 and 19.6 min, respectively. Their
structures were proposed as 1-methylindole N-demethylated arbidol and 4'-hydroxylarbidol,
respectively. Based on the high collision energy mass spectra, the chemical structure of M6-2
was further confirmed by comparison with a reference standard. Arbidol was the predominate
component excreted in feces, accounting for 32.4% of the dose, and the major metabolite in
feces was the sulfate conjugate of arbidol (M10), accounting for 3.0% of the dose. The other
oxidative metabolites (M5, M6-1, and M8) represented <2% of the dose.

Plasma. A total of 16 metabolites were detected in the human plasma extracts, and M6-1
was the major drug related component, followed by the unmetabolized arbidol. The other
metabolites that presented with relatively high levels in human plasma were M5 and M8.
Metabolite M1, M3-2, and M7 were observed in plasma as minor phase I metabolites. These
oxidative metabolites could be further metabolized to form phase II conjugates (M9-2, M11-2,
M14-1, M15, M16, M20-1, M20-2, and M22). The glucuronide conjugate (M18) and the sulfate conjugate (M10) of the parent drug were also detected in plasma. All of these conjugates were detected as minor metabolites.

**Pharmacokinetics of Arbidol and Metabolites M5, M6-1, and M8.** Table 2 presents the pharmacokinetic parameters determined by noncompartmental analysis. The mean plasma concentration versus time profiles for arbidol, M5, M6-1, and M8 are shown in Fig. 3. After oral drug administration, arbidol was rapidly absorbed with a mean $t_{\text{max}}$ of 1.38 h. M5 had a comparable $t_{\text{max}}$ at 1.50 h, whereas the maximum plasma concentrations of M6-1 and M8 were reached much later at 13.0 h and 19.0 h, respectively. The mean $t_{1/2}$ values of the metabolites were 26.3, 25.0 and 25.7 h, which were clearly longer than that of arbidol (15.7 h).

The sulfone metabolite M8 had the lowest concentration among the three major circulating metabolites, with a mean $C_{\text{max}}$ of 22.7 ng/ml and a mean metabolite-to-parent AUC$_{0-t}$ ratio ($\text{AUC}_m/\text{AUC}_p$) of 0.5 ± 0.2. The N-demethylsulfinyl compound M5 was the second most abundant metabolite in circulation, with a $C_{\text{max}}$ of 80.5 ng/ml and a moderate $\text{AUC}_m/\text{AUC}_p$ of 0.9 ± 0.3. The sulfinyl metabolite M6-1 was the major circulating species with a $C_{\text{max}}$ of 525 ng/ml and the highest $\text{AUC}_m/\text{AUC}_p$ at 11.5 ± 3.6. In the mean plasma concentration-time profiles of arbidol, a second peak was observed at about 3 h, it was not resulted from the enterohepatic recirculation, but because of the inter-individual variability in $T_{\text{max}}$.

**In Vitro Metabolism of Arbidol. Human microsomes.** Biotransformation of arbidol (at concentrations of 5.0 µM and 50 µM) was investigated in HLMs, HIMs, and HKMs. Five prominent metabolites, namely, M3-2, M5, M6-1, M7, and M8, were formed in HLMs and HIMs (Fig. 4), whereas only trace amounts of M6-1 were detected in HKMs. At the arbidol concentration of 5.0 µM in HLMs, the parent drug was extensively metabolized, and the amounts of metabolites formed followed the order of M3-2 > M5 > M7 > M6-1 > M8. At the arbidol concentration of 50 µM, the yield of M6-1 increased, and this order was changed to M3-2 > M6-1 > M5 > M7 ≈ M8. Similar trends of metabolites formation were observed in HIMs. Omission of NADPH completely abolished the metabolism of arbidol, which indicated that these metabolic processes were NADPH-dependent.
To determine the human liver microsomal stability of arbidol and its major circulating metabolites, the parent drug, M5, M6-1 and M8 were separately incubated with HLMs in the presence of NADPH. It was found that M5, M6-1 and M8 were metabolized mainly via N-demethylation and/or sulfoxidation. The \( \text{CL}_{\text{int}} \) values were calculated to be 116, 23.6, 28.9, and 46.6 ml/min/kg for arbidol, M5, M6-1, and M8, respectively (Table 3). According to the classification criteria (12, 13), M5 and M6-1 were categorized as medium-clearance compounds, whereas arbidol and M8 were high-clearance compounds, and the most labile compound was arbidol, which had a \( \text{CL}_{\text{int}} \) value of 109 ml/min/kg.

Recombinant P450 and FMO isoforms. A series of cDNA-expressed P450s and FMOs were used to evaluate the contribution of individual enzymes to arbidol metabolism. Each isoform showed varying degrees of efficiency in the production of oxidative metabolites (Fig. 5). Among those tested, FMOs were only responsible for sulfoxidation, whereas P450s were not only involved in the formation of M6-1 and M8, but also of M5 and M7. Isoforms that showed elevated levels of M6-1 production included CYP1A2, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5, FMO1, FMO3, and FMO5, with FMO1 as the most efficient isoform. The same array of P450s also contributed to the formation of M5, M7 and M8. P450 isoforms such as CYP1A1, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, and CYP4A11 were not involved in the metabolism of arbidol. In adult humans, FMO1 is primarily found in the kidney and intestines (14). Based on the microsomal incubation experiments, the FMO1 contribution to the formation of M6-1 was mainly attributed to the FMO1 expressed in the intestine, not in the kidney. The kinetic parameters for arbidol metabolism by P450s and FMO3 were scaled to a typical human liver, and it was calculated that CYP3A4 showed the highest %TNR in the formation of sulfoxidation and N-demethylation metabolites (Table 4).

Inhibition studies. The effects of CYP inhibitors and heat inactivation on the formation of oxidative metabolites (M5, M6-1, M7, and M8) at 5.0 µM and 50 µM arbidol in HLMs and HIMs are shown in Figs. 6 and 7. In HLMs, when the substrate concentration was set at 5.0 µM, 1-ABT inhibited the formation of M5, M7, and M8 by >90% and that of M6-1 by 39%. Little or no significant inhibition was observed with other inhibitors and heat treatment, with
the exception of ketoconazole, which potently inhibited the formation of M5, M7, and M8 by 81%, 97%, and 93%, respectively. By contrast, the formation of M6-1 increased by 185%. At 50 µM arbidol, 1-ABT inhibited the formation of the four metabolites by >90% in HLMs. Heat treatment inhibited the formation of M5, M7, and M8 by approximately 25%. Under the same conditions, the metabolism of benzydamine, an FMO probe substrate, was inhibited by 82%. Among the five specific P450 inhibitors, ketoconazole significantly reduced the formation of M6-1 by 69%, whereas α-naphthoflavone, ticlopidine, quinidine, and clomethiazole inhibited the formation of M6-1 by 46% to 51%. Similar inhibitory effects were observed for M5, M7, and M8, with ketoconazole as the most potent inhibitor. In HIM incubations, 1-ABT inhibited the formation of four metabolites by approximately 90% regardless of the arbidol concentration. Heating the HIMs for 5 min at 45°C prior to the incubation decreased M6-1 formation by about 20%, and the formation of M5, M7, and M8 by 30 to 65%.

**Discussion**

In the present study, the metabolism and excretion of arbidol were investigated in healthy male volunteers after a single oral administration of 200 mg of arbidol hydrochloride. This study is the first to report on the arbidol metabolites in human plasma and feces. A total of 31 metabolites were found and identified in urine, 24 in feces, and 16 in plasma. Arbidol was mainly excreted in urine as phase II conjugates. The major urinary metabolites were glucuronide arbidol (M18) and glucuronide sulfinylarbidol (M20-1 and M20-2), which accounted for approximately 3.6% of the dose. The urinary excretion of sulfate conjugates (M10, M11-2, M14-1, and M15) was estimated to be 2.7% of the dose. The urinary excretion of parent drug was less than 0.1% of the dose. Similar to preclinical species (1), about 32.4% of the total intake dose of arbidol was excreted unchanged within 0–96 h via feces. The sulfate conjugate of arbidol (M10) was the major metabolite in human feces, accounted for 3.0% of the dose, and other metabolites (M5, M6-1, M8) represented <2% of the dose. Because the reference materials for the conjugated metabolites were not available, the amounts excreted of these phase II metabolites were semi-quantified using arbidol as
calibration standard. Further experiments should be carried out using radiolabeled compound
to accurately evaluate the excretion of arbidol in humans after oral drug administration.

It has long been known that circulating metabolites may contribute to drug efficacy and
side effects, and attention should be paid to metabolites that are formed at greater than 10% of
parent drug systemic exposure (February, 2008 FDA Guidance for Industry, Safety Testing of
Drug Metabolites. Pharmacology and Toxicology; http://www.fda.gov/cder/guidance/). After
the oral administration of arbidol hydrochloride to healthy volunteers, M6-1 was detected as
the major circulating species in the plasma, followed by the parent drug, M5, and M8. The
mean elimination half-lives of the three metabolites were longer than that of the parent drug.

Although the average plasma concentrations of M5 and M8 were much lower than that of
arbidol, their AUC$_{0-\infty}$ values were equivalent or comparable to that of the parent, and the
values of AUC$_{m}$/AUC$_{p}$ were 0.9 ± 0.3 and 0.5 ± 0.2 for M5 and M8, respectively. The
exposure of M6-1 in human plasma as determined by AUC$_{0-\infty}$ and C$_{\text{max}}$ were 12.9- and
1.12-fold greater than those of unchanged arbidol. Multiple factors could contribute to the
pharmacokinetic properties of M6-1, including its formation, stability in the circulation, tissue
binding, and effects of transporters in liver and intestine. Assessing the safety and efficacy of
M6-1 is important because of its high exposure and long elimination half-life. A search of the
literature resulted in a single patent which reported that M6-1 could inhibit protein kinase C
(IC$_{50}$ = 7.78 µM) (15). It was speculated that during arbidol treatment, metabolite M6-1 may
account for some of the pharmacological activities associated with arbidol, and measuring
arbidol concentrations along may underestimate the potency and duration of effect for this
agent. In addition, the recommended dosage of arbidol for the treatment of influenza is 200
mg three times daily for 5 to 10 days, and the extended half-life of M6-1 suggested that it
would accumulate on repeated daily dosing of arbidol. Further investigation is needed to
understand the importance of M6-1 in terms of safety and efficacy.

A comprehensive set of in vitro experiments was conducted to investigate the
biotransformation of arbidol (at 5.0 µM and 50 µM). It was found that arbidol was
metabolized by HLMs and HIMs, but not by HKMs, which suggested that liver and intestines
could be the major organs that metabolize arbidol in humans. HLM stability analysis revealed that the CLint of arbidol was much higher than that of M5, M6-1, and M8, which may partially explain the relatively longer plasma t1/2 of M5, M6-1, and M8 compared with that of the parent drug.

It was reported that the major monooxygenases that catalyze the formation of aliphatic sulfoxides are the P450s and FMOs (16-21). Identification of the human P450 and FMO enzymes involved in arbidol metabolism was carried out using isoform screening assays. The results indicated that multiple enzymes, including CYP1A2, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5, FMO1, FMO3, and FMO5 were capable of metabolizing arbidol. However, FMOs were only involved in arbidol sulfoxidation. To better estimate the contribution of each enzyme to the overall metabolism of arbidol in HLMs, the activities of P450s and FMO3, the major FMO isoform in human liver (22), were normalized to the content of each enzyme in HLMs (Table 4). The results indicated that CYP3A4 was the most active enzyme involved in arbidol metabolism, followed by FMO3 (only catalyzed the formation of M6-1), CYP2E1, CYP1A2, CYP2D6, CYP2C9, and CYP3A5. 1-ABT and heat pretreatment of HLMs and HIMs were used to differentiate the contributions of P450s and FMOs to the metabolism of arbidol in humans. At a low arbidol concentration (5.0 µM), incubation of HLMs in the presence of 1-ABT decreased the formation of M5, M7, and M8 by >90%, and that of M6-1 by 39%. By contrast, mild heat treatments, known to significantly reduce FMO activity, weakly affected the arbidol metabolism in HLMs. These results indicated that arbidol metabolism was predominantly P450-driven as compared with FMOs. Furthermore, P450 chemical inhibition studies revealed that inhibition of CYP3A4 with ketoconazole decreased the production of M5, M7, and M8 by >80%, and this could be the reason for the corresponding increase of M6-1 (to 185%), because the secondary metabolism of M6-1 was inhibited by ketoconazole, and other P450s and FMOs catalyzed the formation of M6-1 compensatory. At a high arbidol concentration (50 µM), similar trends were observed in the inhibitory effects of 1-ABT and heat treatments (90% versus 25%). Among the five P450 inhibitors, ketoconazole showed the most potent inhibitory effect. In contrast to the
results obtained at low substrate concentration, other P450 chemical inhibitors exhibited inhibitory effects to a certain degree. This result could be attributed to the saturation of multi-P450 mediated arbidol metabolism at high substrate concentration, and consequently, blocking the function of a single enzyme reduced metabolites formation obviously. When arbidol was incubated with HIMs, both 1-ABT and heat treatment inhibited metabolites formation, and the inhibitory effects of 1-ABT was always greater than that of the heat treatment (Fig. 7). Therefore, P450s are the major enzymes involved in the arbidol metabolism in human intestines, with FMOs contributing to a lesser degree. Overall, the in vitro studies indicated that P450s mainly catalyzed arbidol metabolism by HLMs and HIMs compared with FMOs, with CYP3A4 as the most active enzyme. It could be predicted that there was metabolic DDI potential between arbidol and coadministrated drugs that are CYP3A4 inhibitors or inducers.

In conclusion, arbidol undergoes extensive phase I and phase II metabolism in humans. New metabolic pathways have been proposed, including N-demethylation of 1-methylindole, 4′-hydroxylation, oxidative S-dealkylation, and di-N-demethylation. The study extended the available information on the pharmacokinetics of arbidol metabolites in the circulation. Sulfinylarbidol (M6-1) is the most abundant component in human plasma, with an extended T_{max}, prolonged elimination half-life, and higher exposure compared with the parent drug. The in vitro studies indicated that CYP3A4 is the major enzyme involved in arbidol metabolism in liver and intestines, with minor contributions from other P450 and FMO enzymes. Arbidol may potentially interact with CYP3A4 inhibitors and inducers. Overall, the in vivo and in vitro findings provided new insights into the metabolism and disposition of arbidol in humans.

Acknowledgments

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References


Legends for figures

Fig. 1. Metabolic profiles of arbidol after a single oral administration of 200 mg-arbidol hydrochloride capsules. (A) Pooled plasma sample collected at 2 h postdose; (B) Pooled urine samples collected at 0-24 h period postdose; (C) Pooled feces samples collected at 24-96 h postdose. The left three panels represent metabolite profiles by MS detection, and the right three panels represent metabolite profiles by UV detection at 316 nm.

Fig. 2. Identified metabolic processes of arbidol in humans. The major metabolic pathway was sulfoxidation. Details of chemical structures are shown in Table 1.

Fig. 3. Mean plasma concentration-time profiles of arbidol, M5, M6-1, and M8 after a single oral administration of 200 mg-arbidol hydrochloride capsules to 4 healthy male subjects. (A) linear scale and (B) semi-logarithmic scale.

Fig. 4. Metabolic profiles of arbidol in human liver (A) and intestine (B) microsomes incubations at 5.0 and 50 µM arbidol. The incubations were for 60 min at 1 mg/ml protein and 37°C.

Fig. 5. Formation of M5, M6-1, M7, and M8 in incubations of arbidol (5.0 or 50 µM) with human cDNA-expressed P450s and FMOs. The incubations were performed at 50 pmol of P450/ml and at 4 pmol of FMO/ml.

Fig. 6. Inhibition of formation of M5, M6-1, M7 and M8 in the incubations of arbidol (5.0 or 50 µM) in HLMs by P450 inhibitor(s) and heat treatment. %Remaining activity = metabolic activity in the presence of inhibitor/metabolic activity in microsomes. Two-sided t test analyses: *, P<0.01; **, P<0.001, compared with the control value.

Fig. 7. Inhibition of formation of M5, M6-1, M7 and M8 in the incubations of arbidol (5.0 or
546  50 µM) in HIMs by the P450 inhibitor 1-aminobenzotriazole and heat treatment. %Remaining
547  activity = metabolic activity in the presence of inhibitor/metabolic activity in microsomes.
548  Two-sided t test analyses: *, P<0.01; **, P<0.001, compared with the control value.
Table 1 Metabolites for arbidol in human plasma, urine and feces after oral drug administration (P-plasma; U-urine; F-feces). 

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<th>Chemical Formula</th>
<th>Proposed Chemical Structure</th>
<th>Rt (min)</th>
<th>m/z [M + H]^+</th>
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<td></td>
<td>671.091$^b$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>495.059</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>M22</th>
<th>C$<em>{28}$H$</em>{33}$BrN$<em>2$O$</em>{11}$S</th>
<th>11.2</th>
<th>685.105$^a$</th>
<th>685.107$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(P, U, F)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>685.105$^a$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>685.107$^b$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>509.071, 464.011, 419.988</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2  Pharmacokinetic parameters of arbidol and its three major metabolites in plasma of four healthy male subjects after a single oral administration of 200 mg of arbidol hydrochloride

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Arbidol</th>
<th>M5</th>
<th>M6-1</th>
<th>M8</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{\text{max}}, , \text{h}$</td>
<td>$1.38 \pm 1.11$</td>
<td>$1.50 \pm 1.00$</td>
<td>$13.0 \pm 8.2$</td>
<td>$19.0 \pm 14.0$</td>
</tr>
<tr>
<td>$C_{\text{max}}, , \text{ng/ml}$</td>
<td>$467 \pm 174$</td>
<td>$80.5 \pm 37.5$</td>
<td>$525 \pm 147$</td>
<td>$22.7 \pm 9.8$</td>
</tr>
<tr>
<td>$AUC_{0-t}, , \text{ng} \cdot \text{h} \cdot \text{ml}^{-1}$</td>
<td>$2103 \pm 614$</td>
<td>$1743 \pm 466$</td>
<td>$23104 \pm 4829$</td>
<td>$1040 \pm 483$</td>
</tr>
<tr>
<td>$AUC_{0-\infty}, , \text{ng} \cdot \text{h} \cdot \text{ml}^{-1}$</td>
<td>$2203 \pm 691$</td>
<td>$2121 \pm 546$</td>
<td>$28399 \pm 7656$</td>
<td>$1315 \pm 561$</td>
</tr>
<tr>
<td>$t_{1/2}, , \text{h}$</td>
<td>$15.7 \pm 3.8$</td>
<td>$26.3 \pm 5.9$</td>
<td>$25.0 \pm 5.4$</td>
<td>$25.7 \pm 8.8$</td>
</tr>
<tr>
<td>CL/F, L/h</td>
<td>$99 \pm 34$</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3 *In vitro* $t_{1/2}$ and intrinsic clearance ($CL_{int}$) values for arbidol, M5, M6-1, and M8 in HLMs

<table>
<thead>
<tr>
<th>Compound</th>
<th>$t_{1/2}$ (min)</th>
<th>$CL_{int}$ (ml/min/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arbidol</td>
<td>8.20 ± 0.29</td>
<td>116 ± 3.7</td>
</tr>
<tr>
<td>M5</td>
<td>37.0 ± 2.0</td>
<td>23.6 ± 1.3</td>
</tr>
<tr>
<td>M6-1</td>
<td>30.3 ± 3.2</td>
<td>28.9 ± 2.9</td>
</tr>
<tr>
<td>M8</td>
<td>18.7 ± 0.6</td>
<td>46.6 ± 1.5</td>
</tr>
</tbody>
</table>
Table 4 Total normalized rate of the formation of M5, M6-1, and M8 by individual P450 and FMO isoforms.

<table>
<thead>
<tr>
<th>Isoforms</th>
<th>Mean Content in Human Liver&lt;sup&gt;a&lt;/sup&gt; (pmol isoform/mg protein)</th>
<th>Formation Rate (pmol/min/mg)</th>
<th>Normalized Rate (pmol/min/mg)</th>
<th>Total Normalized Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean content data were obtained from Rodrigues (1999).</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CYP1A2 45.0</td>
<td>0.11</td>
<td>0.31</td>
<td>5.01</td>
</tr>
<tr>
<td></td>
<td>CYP2C19 19.0</td>
<td>0.04</td>
<td>0.18</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>CYP2D6 10.0</td>
<td>0.21</td>
<td>0.75</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>CYP2E1 49.0</td>
<td>0.08</td>
<td>0.31</td>
<td>3.76</td>
</tr>
<tr>
<td></td>
<td>CYP3A4 108</td>
<td>0.20</td>
<td>0.57</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>CYP3A5 1.00</td>
<td>0.09</td>
<td>0.34</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>FMO1 N.A.</td>
<td>2.66</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FMO3&lt;sup&gt;b&lt;/sup&gt; 80.0</td>
<td>0.57</td>
<td>45.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FMO5 N.A.</td>
<td>0.15</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N.A., not available.

<sup>a</sup> Mean content data were obtained from Rodrigues (1999).

<sup>b</sup> Mean content data for FMO3 was obtained from BD Gentest (2003 product catalog).