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Metabolite identification of arbidol in human urine by the study of CID fragmentation pathways using HPLC coupled with ion trap mass spectrometry

Yuya Wang, Xiaoyan Chen, Qiang Li and Dafang Zhong*

Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 646 Songtao Road, Shanghai 201203, China

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The metabolism of arbidol in humans was studied using liquid chromatography-electrospray ionization (ESI) ion trap mass spectrometry (ITMS) after an oral dose of 300-mg arbidol. A total of 17 metabolites were identified including the glucuronide arbidol and the glucuronide sulfinylarbidol as the major metabolites.

Arbidol and its metabolites have some common fragmentation patterns as a result of a homolytic bond cleavage. This cleavage will form odd-electron ions with the loss of a radical. The arbidol fragmentation sequence is first to lose dimethylamine (45 Da), followed by the loss of acetaldehyde (44 Da), and then the phenylthio radical (109 Da). This fragmentation sequence is also observed from *N*-demethylarbidol, sulfonylarbidol, and *N*-demethylsulfonylarbidol. However, for sulfinylarbidol and *N*-demethylsulfinylarbidol, the fragmentation sequence is reversed so that the phenylsulfiny radical (125 Da) was lost first, followed by the loss of dimethylamine (45 Da), and then acetaldehyde (44 Da). The exact masses for arbidol and sulfinylarbidol fragment ions were determined by a quadrupole/time-of-flight mass spectrometer (Q-TOF MS).

The phase II metabolites, such as sulfate and glucuronide conjugates of arbidol, *N*-demethylarbidol, sulfonylarbidol, and *N*-demethylsulfonylarbidol were identified by observing the neutral loss of 80 Da (SO_3) or 176 Da (glucuronic acid) from the MS^2 spectra. The sulfate and glucuronide conjugates such as sulfinylarbidol and *N*-demethylsulfinylarbidol had an unusual fragmentation pattern, in which the phenylsulfinyl radical (125 Da) was lost before the loss of SO_3 group (80 Da) or glucuronic acid (176 Da) occurred.

KEYWORDS: arbidol; metabolite identification; fragmentation; $ITMS^{(n)}$; conjugates

INTRODUCTION

Arbidol is an antiviral active chemical entity created by the Chemical Drug Center of All Russian Research Institute of Pharmaceutical Chemistry together with the Institute of Medical Radiology.[1] Early reports showed that arbidol inhibits influenza virus–induced membrane fusion and may have the capacity to induce serum interferon.[2,3] In Russia, arbidol has been used for the treatment of influenza for several years.[4] Recent studies showed that arbidol exhibits inhibitory activity against other human viruses such as the respiratory syncytial virus, parainfluenza virus 3, rhinovirus 14, and hepatitis B virus.[5,6] Bird viruses such as the avian coronavirus and the H5/N1 influenza A virus were also shown to be sensitive to arbidol.[7,8] Arbidol is now considered as a broad-spectrum antiviral compound and is widely used for the treatment of influenza and some other kinds of respiratory infections because of its fair safety and efficacy.[9]

As arbidol becomes more widely used, there is a strong demand to better understand the parent compound, as well as its metabolic pathways in humans. The physiological mechanism of arbidol has been reported to activate the phagocytic activity of macrophages, and stimulate some forms of cellular and humoral immunity.[9] The pharmacokinetics' studies in animals have shown that arbidol is absorbed and distributed quickly into tissues and organs after oral administration.[10] The quantitative determination of arbidol in human plasma has also been reported[11,12] in recent years.

The metabolic behavior of arbidol has been scarcely studied. Literature search resulted in a single article published in 1995.[1] The metabolites of arbidol from rat urine were identified by the use of thin-layer chromatography (TLC/LC) coupled with mass spectrometry.[1] No human metabolic study of arbidol has been reported so far.

In this article, we will report the metabolites of arbidol identified from human urine with the use of high performance liquid chromatography (HPLC) coupled with an ion trap mass spectrometer (ITMS).

Liquid chromatography coupled with mass spectrometry (LC–MS) has been widely accepted as the ideal analytical technique for metabolic characterization.[13] Traditional approach for the metabolite identification was with the use

*Correspondence to: Dafang Zhong, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 646 Songtao Road, Shanghai 201203, China. E-mail: dfzhong@mail.shcnc.ac.cn

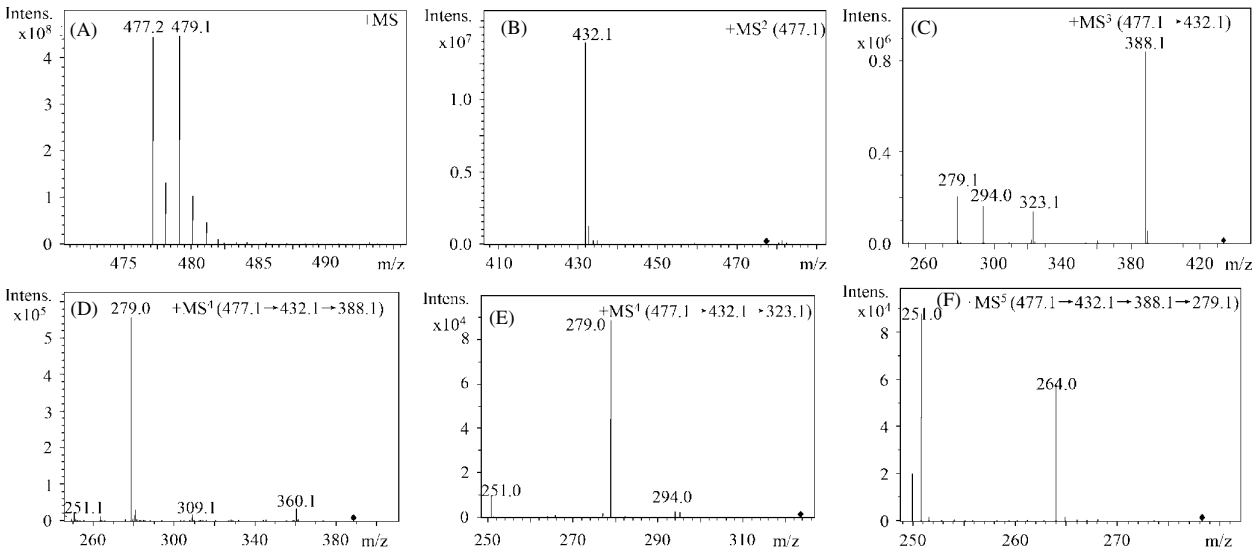

Figure 1. MS (A), MS^2 (B), MS^3 (C), MS^4 (D and E) and MS^5 (F) spectra of arbidol.

of triple quadrupole mass spectrometry. After initial full-scan screening, the potential metabolites identified can be further confirmed along with possible sites of biotransformation identified by performing precursor ion scan, product-ion scan, as well as neutral loss scan. The disadvantage of this approach is that a single MS/MS experiment is usually insufficient for localizing the site of biotransformation. An ITMS is then an attractive choice since it can be used to narrow down the site of biotransformation by studying the fragmentation patterns from the MS^n experiments.[13]

In this work, a sensitive and specific LC-electrospray ionization (ESI)-ITMS method has been developed for the identification of arbidol and its metabolites in human urine. Meanwhile, fragmentation sequences of arbidol and its metabolites were investigated so that the collision-induced dissociation (CID) pathways were deduced in detail even though these compounds were structurally closely related analogs. Important major fragment ions were also positively confirmed from the exact mass measurement by using the HPLC/Q-TOF MS (quadrupole/time-of-flight mass spectrometer).

EXPERIMENTAL

Reagents and materials

The arbidol reference (purity above 98%) was provided with the courtesy of Professor Ping Gong from Shenyang Pharmaceutical University (Liaoning, China). The HPLC grade methanol was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ammonium acetate of HPLC grade was purchased from E. Merck (Darmstadt, Germany). Distilled water was obtained in-house from Millipore Milli-Q water purification system (Millipore, Bedford, MA, USA). Sulfinylarbidol and sulfonylarbidol with >98% purity (based on HPLC/MS analysis) were synthesized in our laboratory. Their structures were identified by positive ESI–MS^n, ^{1}H NMR and ^{13}C NMR.

The 10 μg/ml stock solution of arbidol was prepared in methanol.

Analytical instrumentation

HPLC-ITMS experiment was carried out on an Agilent 6300 LC/MSD Trap XCT ultra (Agilent Technologies, Waldbronn, Germany).

Chromatography

The Agilent 1200 HPLC was equipped with a reversed-phase column (Ultimate XB-C_{18}, 4.6 mm × 150 mm i.d., 5 μm, Welch Materials, USA) protected by a 4.0 mm × 3.0 mm i.d. Security Guard (5 μm) C_{18} guard column (Phenomenex, Torrance, CA, USA). The mobile phase was a gradient of a mixture of (A) methanol and (B) 5-mM ammonium acetate programmed as follows: initial 30% A maintained for 15 min, then increased to 50% in 0.5 min; maintained at 50% for 10 min, then increased to 70% in 0.5 min; maintained at 70% for 15 min, and finally decreased to 30% A in 0.5 min, and maintained at 30% A for 10 min. The flow rate was 0.6 ml/min and the injection volume was 20 μl.

The ion trap mass spectrometer

The mass spectrometer (MSD) was equipped with an ESI source. The ionization mode was positive. The interface and MSD parameters were as follows: nebulizer pressure 40 psi (N_2), dry gas 12 ml/min (N_2), dry gas temperature 350 °C, spray capillary voltage 3500 V, skimmer voltage 40 V, ion transfer capillary exit 124 V, scan range m/z 150–800, spectra average 3, target 200 000, and dwell time 200 ms. For MS^n spectra, the fragmentation amplitude varied between 0.4 and 0.9 V. Other parameters, including the potentials of octapole offset and the tube lens offset, were also optimized for maximum abundance of the ions of interest by the automatic tune procedure of the instrument. The MS^n product-ion spectra were produced by CID of the molecular ion $[M + H]^+$ of all analytes at their respective HPLC retention times. Data acquisition was performed in full-scan LC/MS and

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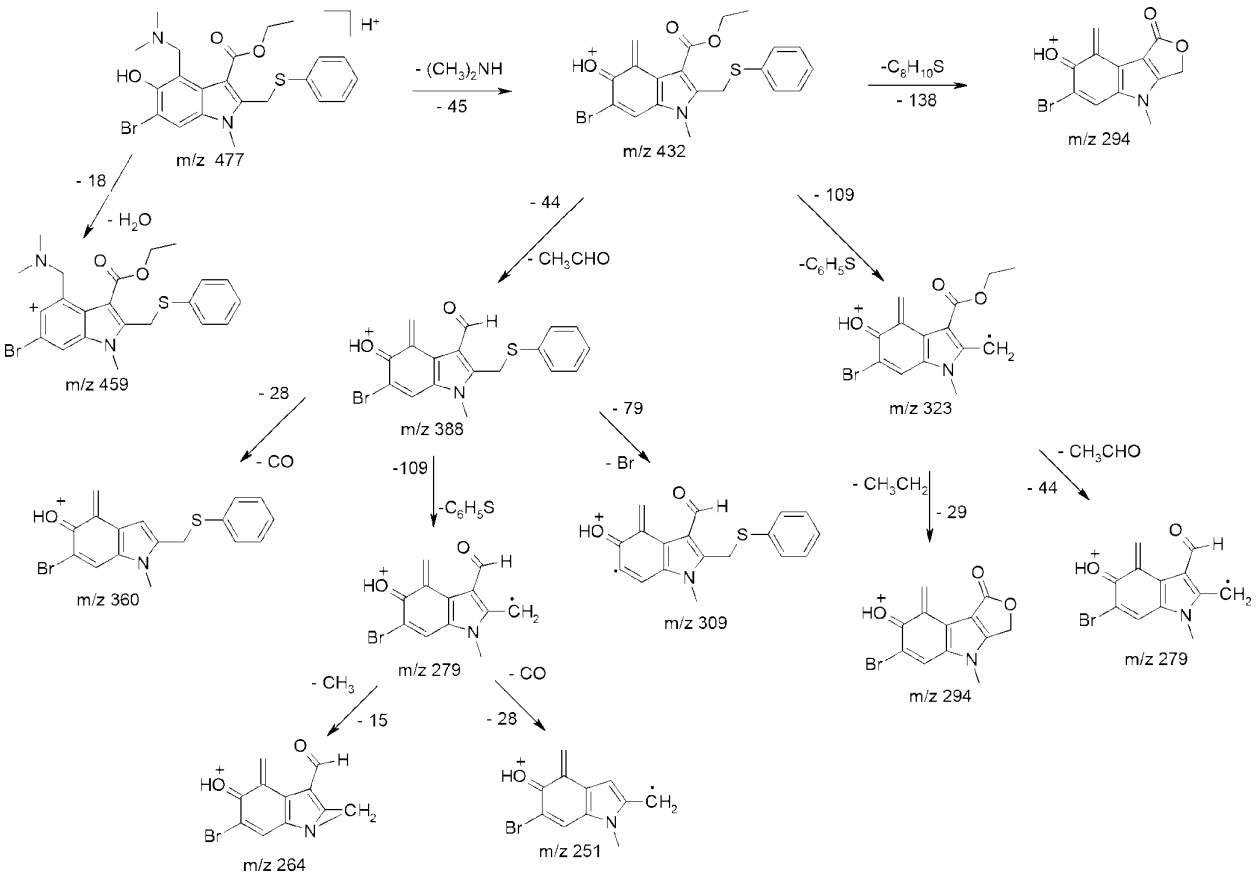

Figure 2. Proposed CID pathway of arbidol at the precursor of *m/z* 477.

MS^n modes. All data acquired were processed by Agilent Chemstation Rev. B. 01.03 software (Agilent, Palo Alto, CA, USA).

The Q-TOF MS

The high mass resolution experiments were carried out on an Ultima Global tandem Q-TOF MS (Waters Corporation, Manchester, UK). The ESI was operated using nitrogen as the desolvation and nebulizing gas at a temperature of 150 °C. The spray capillary and the cone voltages were set to 2650 and 100 V, respectively. The cone block was heated to 120 °C and was set to a potential between 80 and 100 V. For CID experiments, argon was used as the collision gas and the collision energy was set to 55 V. Instrument control, data acquisition, and processing were performed with a MassLynx data system (Version 4.0).

Urine sample collection

Arbidol capsules (Shijiazhuang No. 4 Pharmaceutical Co., Ltd, Heibei, China) used for this work were purchased from a regular pharmacy as arbidol is an over-the-counter drug in China. The arbidol capsules were given to two nonsmoking male volunteers (both with written informed consents). The arbidol urine samples were collected within a period of 0–24 h. The blank urine samples were collected prior to dosing. The urine samples were frozen to −20 °C immediately after the collection. None of the subjects were taking any continuous medications.

Sample preparation

To a 200-μl aliquot of urine sample, a 400-μl methanol was added. This mixture was vortex-mixed and centrifuged at 11 000 *g* for 5 min. The supernatant was transferred into a glass tube so that it was evaporated to dryness under a stream of nitrogen. The reconstitution solution was 100 μl of methanol and 5-mM ammonium acetate (30 : 70, v/v).

Urine treated by the β-glucuronidase

A 2000 unit β-glucuronidase (Type HA-4, Sigma Chemical Co., St. Louis, MO, USA) was used for the hydrolysis of glucuronide conjugates. The incubation was performed for two samples, one was blank urine and the other was arbidol urine. For the incubation, 200-μl aliquots of urine were mixed with 200 μl of β-glucuronidase (in 1 M citrate buffer solution at pH 5.0). The incubation was carried out at 37 °C for 16 h. The effect of the glucuronidase was studied by comparing the peak intensities for compounds of interests before and after the incubation. The compounds of interest included glucuronide conjugates and their nonglucuronidated forms (hydrolyzed form).

RESULTS AND DISCUSSION

Proposed CID pathways of the parent drug arbidol

To study the metabolic behavior of a new compound, one of the very first step is to fully understand the

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Table 1. LC/Q-TOF MS and MS^2 data obtained for arbidol and M1

	Measured mass	Calculated mass	Formula	Error mDa	Error ppm	Ion type
Arbidol	477.0847	477.0848	$C_{22}H_{26}N_2O_3SBr$	−0.1	−0.2	Even electron
MS^2 (m/z 477)	432.0259	432.0269	$C_{20}H_{19}NO_3SBr$	−1.0	−2.3	Even electron
	387.9991	388.0007	$C_{18}H_{15}NO_2SBr$	−1.0	−2.3	Even electron
	323.0153	323.0157	$C_{14}H_{14}NO_3Br$	−0.4	−1.3	Odd electron
	293.9763	293.9767	$C_{12}H_{11}NO_3Br$	−0.4	−1.3	Even electron
	278.9880	278.9895	$C_{12}H_{10}NO_2Br$	−1.5	−5.3	Odd electron
M1	493.0797	493.0798	$C_{22}H_{26}N_2O_4SBr$	−0.1	−0.2	Even electron
MS^2 (m/z 493)	368.0717	368.0736	$C_{16}H_{21}N_2O_3Br$	−1.9	−5.0	Odd electron
	323.0153	323.0157	$C_{14}H_{14}NO_3Br$	−0.4	−1.3	Odd electron
	278.9886	278.9895	$C_{12}H_{10}NO_2Br$	−0.9	−3.2	Odd electron

parent compound's mass spectrometric behavior, i.e. its fragmentation pattern.[14] This is important for distinguishing metabolites from the fragment ions of the parent compound. This can also help in identifying metabolites that originated from the dealkylated fragments of the parent compound.

The parent compound arbidol produces doublet m/z 477/499 ions (Fig. 1(A)) because of the presence of Br in the molecule (structure shown in Fig. 2). Bromine has the distinct ^{79}Br and ^{81}Br isotopic pattern.

Figure 1 shows the spectra of the MS^n of the m/z 477 ion obtained from the ion trap experiments. The MS^2 spectrum of m/z 477 produced ions of m/z 459 and 432 (Fig. 1(B)). The 459 ion was formed by the loss of water, while the 432 ion was formed after the neutral loss of dimethylamine (−45 Da with a proton shift) as a result of the C–N bond cleavage. The MS^3 spectrum of the m/z 432 (477 → 432) showed fragment ions of m/z 388, 323, 294, and 279 (Fig. 1(C)). The m/z 388 and 323 ions were formed by the respective loss of the acetaldehyde (−44 Da) and phenylthio (−109 Da) radical, while m/z 294 was the result of the structural rearrangement to form lactone from m/z 432. The MS^4 spectrum of the m/z 388 ion (477 → 432 → 388) shows fragment ions at m/z 360, 309, 279, and 251 (Fig. 1(D)). The m/z 360, 309, and 279 ions were formed by the loss of CO (−28 Da), bromine radical (−79 Da) and phenylthio radical (−109 Da), respectively. The MS^4 spectrum of the 323 ion (477 → 432 → 323) shows fragment ions of m/z 279 and 251 (Fig. 1(E)). The MS^5 of m/z 279 ion (477 → 432 → 323 → 279) shows fragment ions of m/z 254 and 251 (Fig. 1(F)).

It is characteristic for the structure of arbidol that it losses dimethylamine (−45 Da), acetaldehyde (−44 Da) and phenylthio radical (−109 Da) in sequence. The existence of these fragment ions was later positively confirmed by the exact mass measurement from Q-TOF (Table 1). As a result, the CID pathway of the arbidol was deduced as shown in Fig. 2.

Proposed CID pathways for arbidol metabolites

To effectively identify the metabolites of arbidol, the possible metabolic pathways as well as the possible metabolite structures were first theoretically proposed. This was solely based on the arbidol chemical structure and the fundamental metabolism rules. The second step was to run LC/ITMS in full-scan mode for the blank urine and the arbidol urine. Four injections were made in total: two for blank and arbidol urines before the β-glucuronidase incubation, and two for blank and arbidol urines after the β-glucuronidase incubation. The MS spectra obtained from these four samples were compared so that the possible metabolites could be identified. It is important to pay attention to the in-source decomposition of $[M + H - SO_3]^+$ to avoid obtaining false positives. The existence of the $^{79}Br/^{81}Br$ isotopic pattern was also helpful in identifying the compounds that originated from arbidol.

The extracted ion chromatograms (EICs) of detected arbidol metabolites are shown in Fig. 3(A) and (B). Their LC/MS^n results are listed in Table 2. Comparing the experimentally identified metabolites with those that were theoretically proposed can help to increase our confidence in the findings. The proposed metabolites were then further analyzed by $LC\text{-}ITMS^n$ for structural elucidations. The metabolites' retention times and MS^n mass spectra were compared with what was obtained from arbidol. On the basis of the method mentioned above, the parent compound arbidol and its metabolites were identified from the sample human urine. Their protonated molecules $[M + H]^+$ showed m/z values of 463, 477, 479, 493, 495, 509, 543, 557, 559, 573, 575, 589, 639, 653, 655, 657, 669 and 685, respectively.

Because of the existence of the phenolic hydroxyl group in arbidol, it is expected to find a few glucuronide conjugates from human urine. To further confirm the existence and to help in localizing the site of glucuronization, a β-glucuronidase was used to treat the human urine so that the glucuronide conjugates were hydrolyzed back to their free form. As a result, the LC peak intensities of the glucuronide conjugates were expected to be significantly decreased (or even disappeared) after the treatment of the β-glucuronidase.

In the mean time, the LC peak intensities of the free-form metabolites related to these glucuronide conjugates were expected to be increased.

In addition, the β-glucuronidase we used contained trace active sulfatase, which would cause the sulfate conjugates to be hydrolyzed during the procedure. As a result, after the treatment of β-glucuronidase, the contents of both the glucuronide and sulfate conjugates from the urine samples

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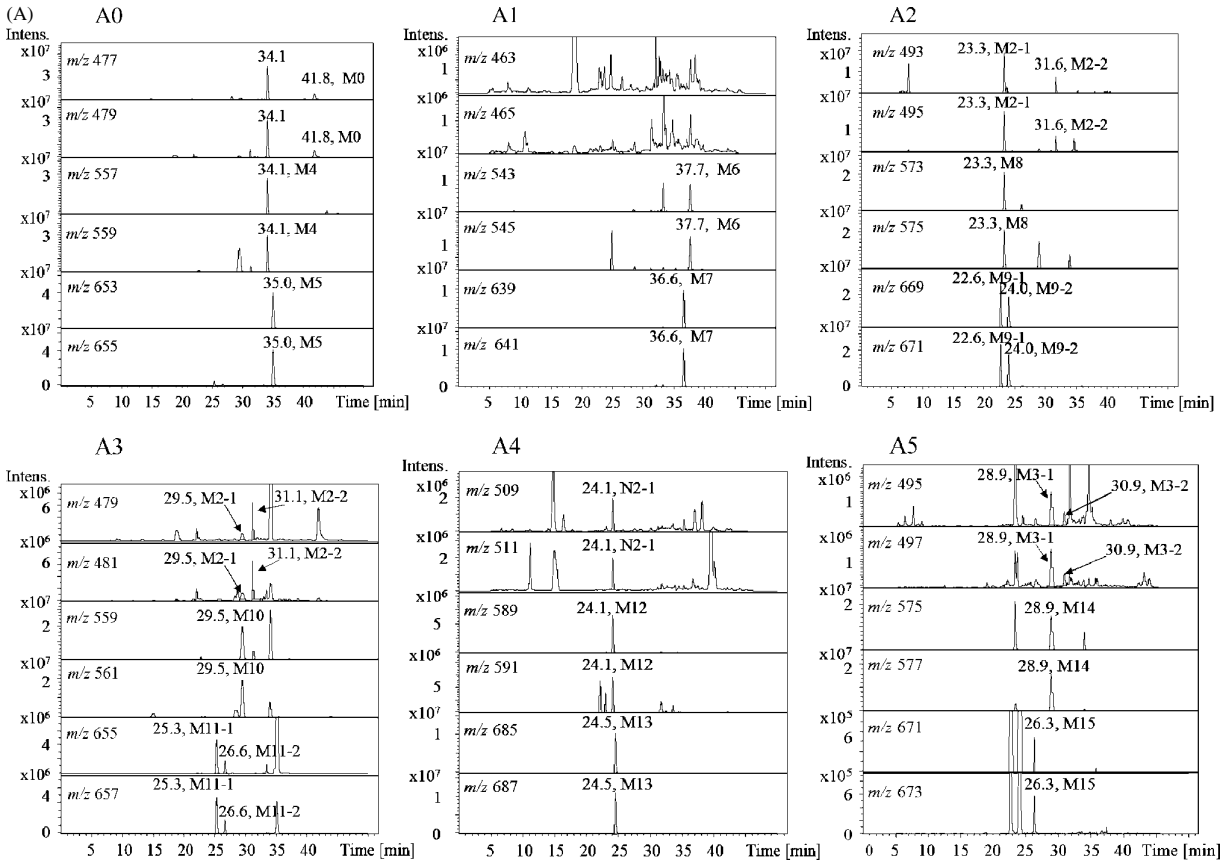

Figure 3. (A) LC/MS ion chromatograms for the metabolites of the free forms, sulfate and glucuronide conjugates, generated using the $[M + H]^1$ and $[M + 2 + H]^1$ ions due to the presence of bromine atom: A0 $[M + H]^1$ and $[M + 2 + H]^1$ of arbidol and its sulfate and glucuronide conjugates (M0, M4 and M5); A1 $[M + H]^1$ and $[M + 2 + H]^1$ of N1 and its sulfate and glucuronide conjugates (N1, M6 and M7); A2 $[M + H]^+$ and $[M + 2 + H]^+$ of M1 and its sulfate and glucuronide conjugates (M1, M8 and M9); A3 $[M + H]^+$ and $[M + 2 + H]^+$ of M3 and its sulfate and glucuronide conjugates (M3, M12 and M13); A4 $[M + H]^+$ and $[M + 2 + H]^+$ of N2 and its sulfate and glucuronide conjugates (N2, M12 and M13); A5 $[M + H]^+$ and $[M + 2 + H]^+$ of M3 and its sulfate and glucuronide conjugates (M3, M14 and M15). (B) LC/MS ion chromatograms for the metabolites of the free forms, sulfate and glucuronide conjugates, generated using the $[M + H]^1$ and $[M + 2 + H]^1$ ions after treatment by β-glucuronidase due to the presence of bromine atom: B0 $[M + H]^+$ and $[M + 2 + H]^+$ of arbidol and its sulfate and glucuronide conjugates (M0 and M4); B1 $[M + H]^+$ and $[M + 2 + H]^+$ of N1 and its sulfate and glucuronide conjugates (M6 and M7); B2 $[M + H]^+$ and $[M + 2 + H]^+$ of M1 and its sulfate and glucuronide conjugates (M1, M8 and M9); B3 $[M + H]^+$ and $[M + 2 + H]^+$ of M2 and its sulfate and glucuronide conjugates (M2, M10 and M11); B4 $[M + H]^+$ and $[M + 2 + H]^+$ of M4 and its sulfate and glucuronide conjugates (N2, M12 and M13); A5 $[M + H]^1$ and $[M + 2 + H]^1$ of M4 and its sulfate and glucuronide conjugates (M3 and M14).

decreased with glucuronide conjugates decreasing at a much greater extent than that of the sulfate conjugates.

The MS^n spectra of arbidol metabolites were studied for detailed structural elucidation. The LC retention time of arbidol standard was found to be 41.8 min. However, a peak at 34.1 min with m/z 477 was also observed from the urine sample. Experiments carried later on showed that this peak was the result of the in-source CID from the arbidol sulfate conjugate. It is critical to be aware of the existence of the sulfate conjugates as well as their related free forms so that ions from the in-source CID were not assigned as new metabolites.

N1

The EICs of m/z 463 (m/z 477 − 14 Da) from the full-scan LC/MS did not show any LC peak. However, an LC peak at m/z 463 with retention time of 38.7 min was observed after the urine sample was treated with β-glucuronidase, and it was named as *N1*. The MS^2 of m/z 463 produced ions at m/z 445 and 432, which were from the loss of H_2O (−18 Da) and the loss of methylamine (−31 Da). The MS^3 spectrum of m/z 432 (m/z 463 → 432) showed fragment ions of m/z 388, 323, 294, and 279. These results indicated that N1 was the demethyl product of arbidol, and the demethylation occurred at the dimethylamine site.

The fact that m/z 463 did not exist prior to the β-glucuronidase treatment indicated that the demethylarbidol existed in human urine only in glucuronide conjugate form, but not the free form. On the basis of these data, N1 was tentatively identified as ethyl-6-bromo-4-[methylamino-methyl]-5-hydroxy-1-methyl-2-[(phenylthio)methyl]-indole-3-carboxylate.

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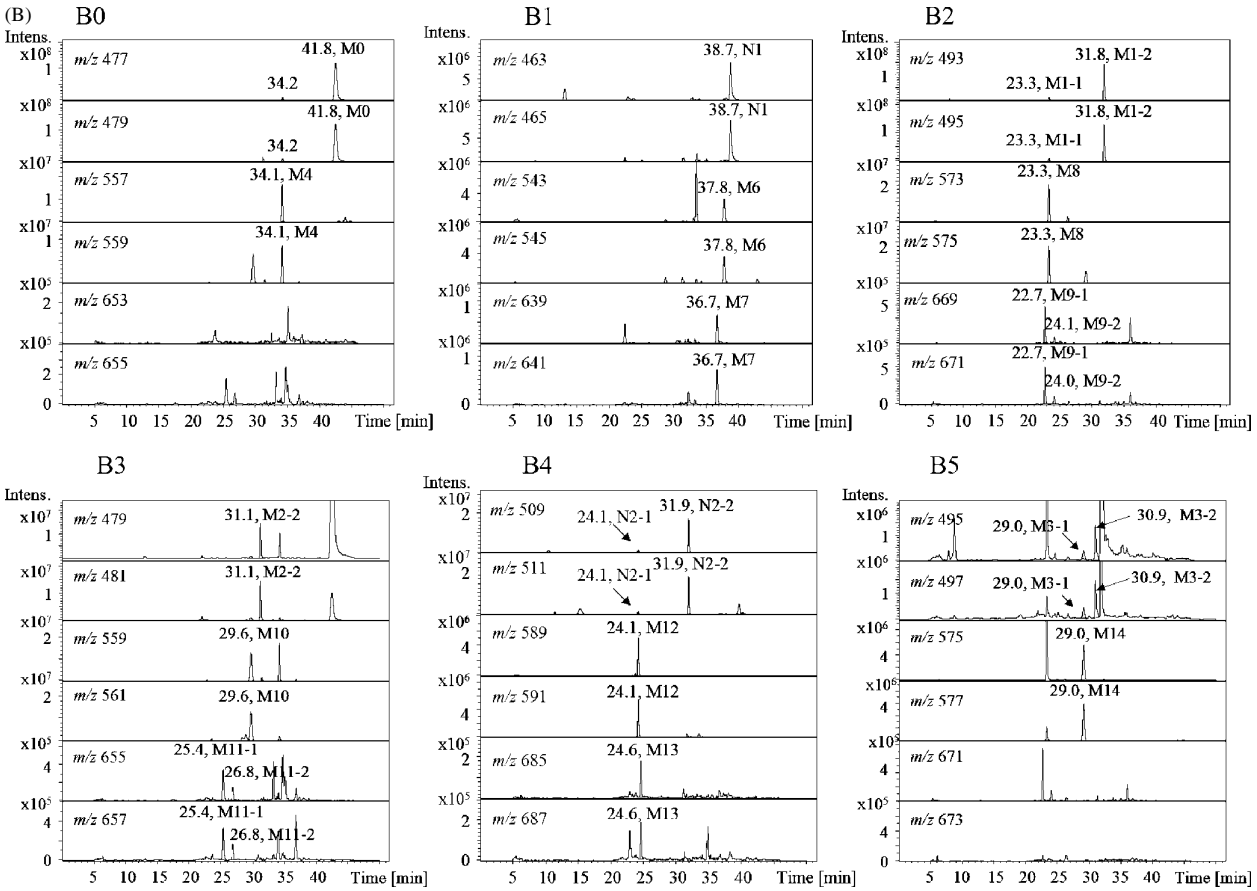

Figure 3. (*Continued*).

M1

The EIC of m/z 493 (m/z 477 + 16 Da) from the full-scan LC/MS showed two LC peaks at 23.3 and 31.6 min (M1-1 and M1-2). After the sample was treated with β-glucuronidase, the intensity of the M1-2 peak increased 20-fold. This is the indication that the intensity increase of M1-2 was the result of the hydrolysis from its glucuronide conjugate. In addition, M1-1 peak intensity decreased a little.

The MS^n spectra of M1-1 and M1-2 showed identical fragment ions. The MS^2 of m/z 493 produced a product ion of m/z 368 (−125 Da) radical. The losing moiety of 125 Da was the phenylthio (109 Da) plus 16. This suggested that the + 16 modification occurred on the phenylthio moiety. The m/z 323 ion was detected in the MS^3 m/z 368 (m/z 493 → 368) as a result of m/z 368 losing the neutral dimethylamine (−45 Da). This m/z 323 ion was a characteristic fragment ion of arbidol. The prominent fragment ion at m/z 279 was detected in the MS^4 of m/z 493 → 368 → 323 by the release of neutral acetaldehyde (−44 Da).

The sequence of the cleavage for M1 was different from that of the arbidol as a result of the phenylthio moiety being oxidized. In other words, the loss of phenylthio moiety (−125 Da) was the first step of fragmentation for this molecule. It is uncommon to have this kind of fragmentation sequence, in which an odd-electron ion is formed first, followed by the loss of a radical as a result of a homolytic bond cleavage, and the neutral groups are lost as a last step. To confirm the existence of these fragment ions, their exact masses were determined by the Q-TOF MS (Table 1). The ions at m/z 368, 323 and 279 were all odd-electron ions, indicating that the proposed mass fragmentation pattern of M1 was correct (Fig. 4).

After comparing with the synthetic reference, M1-2 was confirmed as having the retention time of 31.6 min and was identified as ethyl-6-bromo-4-((dimethylamino)-5-hydroxy-1-methyl-2-[(phenylsulfinyl)methyl]-1*H*-indole-3-carboxylate. More experiments later confirmed that the M1-1 peak was a fragment ion of the M1-2 sulfate conjugate produced by in-source CID. The fact that M1-2 was observed prior to the β-glucuronidase and its LC peak intensity was significantly increased also indicated that this metabolite existed in urine in the free form as well as in the glucuronide conjugate form.

M2

The EIC of m/z 479 (m/z 477 − 14 Da + 16 Da) from the full-scan LC/MS showed two LC peaks at 29.5 and 31.1 min (M2-1 and M2-2). After the sample was treated with β-glucuronidase, the intensity of the M2-2 peak increased twofold. This indicated the existence of the glucuronide conjugates of M2-2. And this peak was observed before and after the β-glucuronidase treatment, which indicated the existence of both the free form and the glucuronide conjugate

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Table 2. LC/MS^n data obtained for arbidol and its metabolites from human urine

	$[M+H]^+$	R_t (min)	MS^n fragments
Arbidol	477, 479	41.9	**MS^2/477:** 459, 432 (100%); **MS^3/432:** 388 (100%), 323, 294; **MS^4/388:** 360, 279 (100%), 309, 251; **MS^4/323:** 279 (100%), 294; **MS^5/279:** 251 (100%), 264
N1	463, 465	38.7	**MS^2/463:** 432 (100%), 445; **MS^3/432:** 388 (100%), 323, 294, 279
M1-1	493, 495	23.3	**MS^2/493:** 368 (100%); **MS^3/368:** 353, 323 (100%); **MS^4/323:** 294, 279 (100%), 251
M1-2	493, 495	31.6	**MS^2/493:** 368 (100%); **MS^3/368:** 353, 323 (100%); **MS^4/323:** 294, 279 (100%), 251
M2-1	479, 481	29.5	**MS^2/479:** 434, 354 (100%), 323
M2-1	479, 481	31.1	**MS^2/479:** 434, 354 (100%), 323
N2-1	509, 511	24.1	**MS^2/509:** 464 (100%), 332, 269; **MS^3/464:** 420 (100%), 323, 294; **MS^4/420:** 295, 279 (100%), 266
N2-2	509, 511	31.9	**MS^2/509:** 464 (100%), 332, 269; **MS^3/464:** 420 (100%), 323, 294; **MS^4/420:** 295, 279 (100%), 266
M3-1	495, 497	29.0	**MS^2/495:** 464 (100%), 420, 332, 279
M3-2	495, 497	30.9	**MS^2/495:** 464 (100%), 420, 332, 279
M4	557, 559	34.0	**MS^2/557:** 477 (100%), 432, 388
M5	653, 655	35.1	**MS^2/653:** 477 (100%), 432, 388, 294
M6	543, 545	37.7	**MS^2/543:** 463, 432 (100%), 388
M7	639, 641	36.6	**MS^2/639:** 463, 432 (100%), 388
M8	573, 575	23.3	**MS^2/573:** 448; **MS^3/448:** 433, 368, 323 (100%)
M9-1	669, 671	22.6	**MS^2/669:** 544; **MS^3/544:** 529, 368, 323 (100%), 279
M9-2	669, 671	24.0	**MS^2/669:** 544; **MS^3/544:** 529, 368, 323 (100%), 279
M10	559, 561	29.4	**MS^2/559:** 434; **MS^3/434:** 419, 354, 323 (100%), 310
M11-1	655, 657	25.3	**MS^2/655:** 530; **MS^3/530:** 515, 486, 354, 323 (100%), 310, 279, 251
M11-2	655, 657	26.9	**MS^2/655:** 530; **MS^3/530:** 515, 486, 354, 323 (100%), 310, 279, 251
M12	589, 571	24.1	**MS^2/589:** 509 (100%), 464, 420
M13	685, 687	24.5	**MS^2/685:** 509 (100%), 464, 420, 294
M14	575, 577	28.9	**MS^2/575:** 495, 464 (100%), 332, 279
M15	671, 673	26.3	**MS^2/671:** 495, 464 (100%), 332, 279

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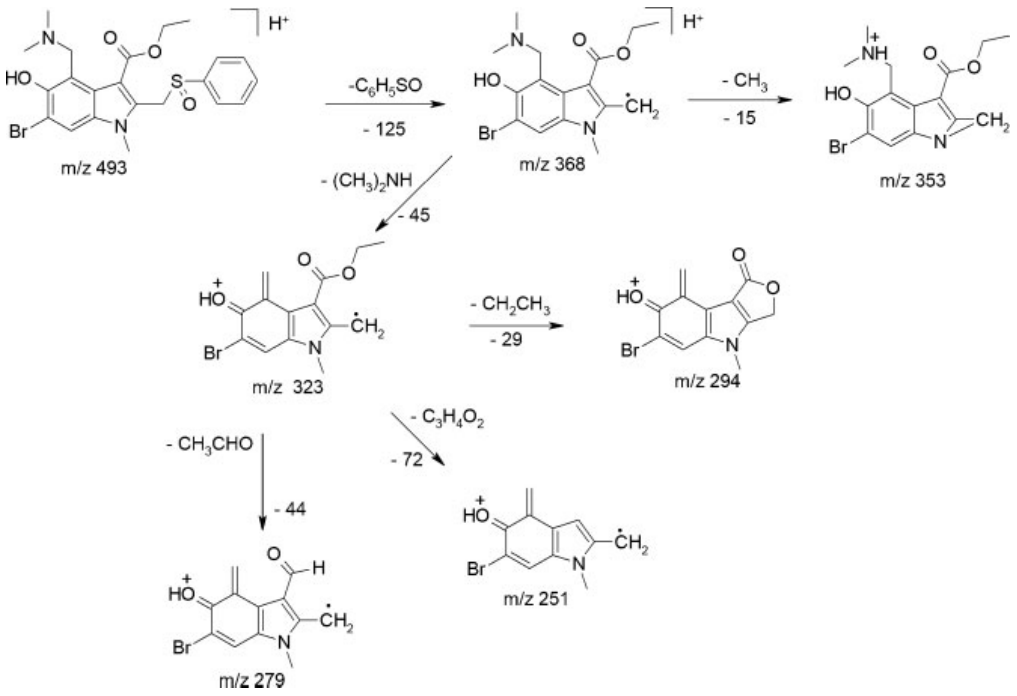

Figure 4. Proposed CID pathway of M1 at the precursor of *m/z* 493.

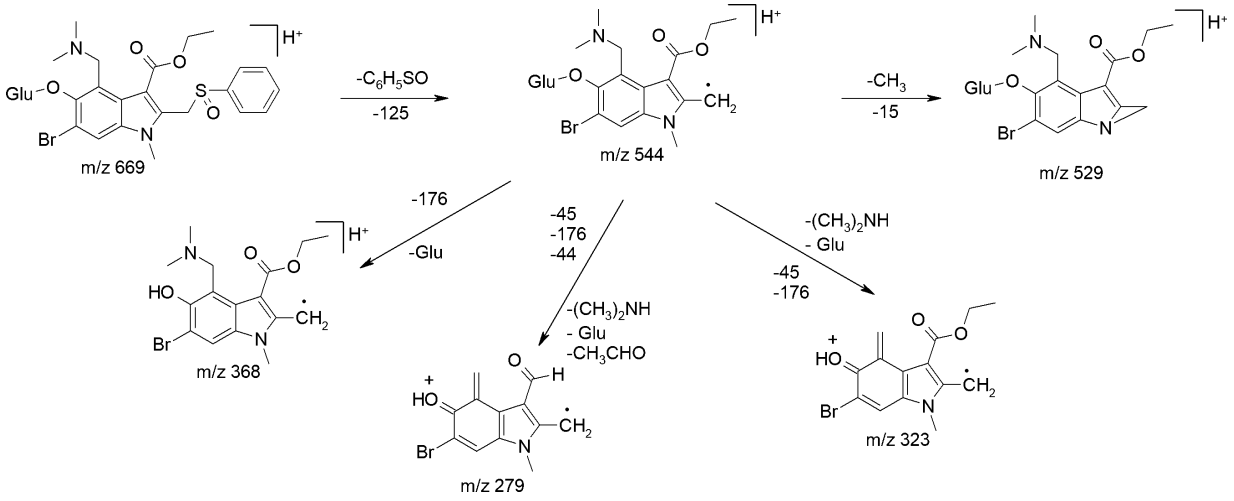

Figure 5. Proposed CID pathway of M9 at the precursor of *m/z* 669.

in human urine. In addition, the M2-1 peak intensity was decreased a little.

The MS^n spectra of M2-1 and M2-2 showed similar fragment ions. The MS^2 spectrum of m/z 479 showed fragment ions at m/z 354 (−125 Da) and 323 (−125 Da − 31 Da). These fragment ions were formed by losing the phenylsulfiny radical (−125 Da) and phenylsulfiny radical (−125 Da) plus one neutral methylamine (−31 Da) respectively. This suggested that the plus 16 modification occurred on the phenylthio moiety and the minus 14 modification occurred on the dimethylamine moiety.

On the basis of these data, M2-2 was tentatively identified as ethyl 6-bromo-4-(methylamino-5-hydroxy-1-methyl-2-[(phenylsulfinyl)methyl]-1*H*-indole-3-carboxylate. More experiments later confirmed that the M2-1 peak was a fragment ion of the M2-2 sulfate conjugate produced by in-source CID.

N2

The EIC of m/z 509 (m/z 477 + 32 Da) from the full-scan LC/MS showed a single LC peak at 24.1 min (N2-1). After the sample was treated with β-glucuronidase, an additional LC peak was observed at 31.9 min (N2-2) with m/z 509. This means that N2-2 did not exist in the free form in the human urine.

The MS^n spectra of N2-1 and N2-2 showed similar fragment ions. The protonated molecule of N2 was m/z 509, suggesting that this metabolite was a dioxygenated derivative of arbidol. The MS^2 of m/z 509 produced product

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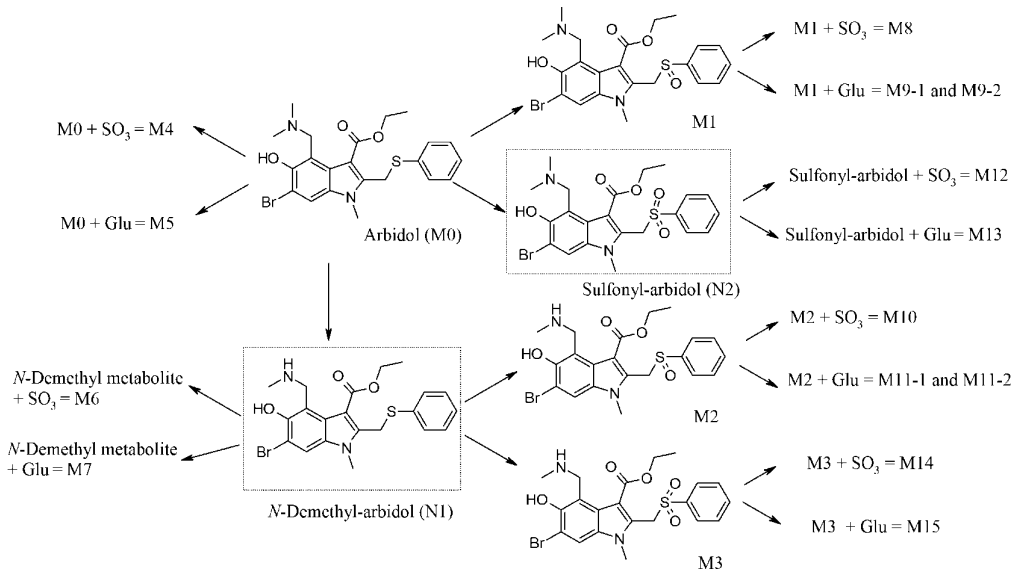

Figure 6. Proposed major metabolic pathways of arbidol in humans.

ion of m/z 464 (−45 Da) radical by losing the neutral dimethylamine. The prominent fragment ions at m/z 420 were detected in the MS^3 m/z 464 (m/z 509 → 464) by the release of neutral acetaldehyde (−44 Da). Furthermore, the prominent fragment ions at m/z 279 were detected in the MS^4 m/z 420 (m/z 509 → 464 → 420). The losing moiety of 141 Da was the phenylthio (109 Da) plus 32. This indicated that the +32 Da modifications occurred on the phenylthio moiety. Although the phenylthio moiety was dioxygenized, the fragment sequence of N2 was the same as that of arbidol.

After comparing with the synthetic reference, N2-2 was identified as ethyl-6-bromo-4-((dimethylamino)-5-hydroxy-1-methyl-2-[(phenylsulfonyl)methyl]-1*H*-indole-3-carboxylate. More experiments later confirmed that the N2-1 peak was a fragment ion of the N2-2 sulfate conjugate produced by in-source CID.

M3

The EIC of m/z 495 (m/z 477 − 14 Da + 32 Da) from the full-scan LC/MS showed two LC peak at 29.0 and 30.9 min (M3-1 and M3-2). After the sample was treated with β-glucuronidase, the intensity of the M3-2 peak increased twofold. This indicated that both the free form and the glucuronide conjugate of M3-2 existed in human urine. In addition, M3-1 peak intensity was decreased a little.

The MS^n spectra of M3-1 and M3-2 showed identical fragment ions. The MS^2 spectrum of M3 showed fragment ions at m/z 464 (−31 Da), 420 (−31 Da − 44 Da) and 323 (−31 Da − 141 Da) by the loss of one neutral methylamine (31 Da), one neutral acetaldehyde (44 Da) and one phenylsulfonyl radical (141 Da). On the basis of these results, M3 was tentatively identified as ethyl 6-bromo-4-(methylamino-5-hydroxy-1-methyl-2-[(phenylsulfonyl)methyl]-1*H*-indole-3-carboxylate. More experiments later confirmed that the M3-1 peak was a fragment ion of the M3-2 sulfate conjugate produced by in-source CID.

M4–M15

M4, M5, M6, M7, M12, M13, M14, and M15

M4 and M5 had a retention time of 34.1 and 35.0 min with m/z values being 557 and 653, respectively. The ions at m/z 557 and 653 were the results of m/z 477 plus 80 and 176, respectively. The MS^2 spectrum of M4 showed fragment ions at m/z 477, 432, 388 as a result of losing of SO_3 (−80 Da), SO_3 plus the neutral dimethylamine (−80 Da − 45 Da), and SO_3 plus dimethylamine plus one neutral acetaldehyde (−80 Da − 45 Da − 44 Da). More attention was paid to the in-source decomposition of $[M + H - 80]^+$ (M1-1) which had the same retention time with M8 for it could interfere with the identification of the metabolite.

The MS^2 spectrum of M5 (m/z 653) showed fragment ions at m/z 477, 432, and 388 as a result of losing glucuronic acid (−176 Da), glucuronic acid plus the neutral dimethylamine (−176 Da − 45 Da), and glucuronic acid plus the dimethylamine plus one neutral acetaldehyde (−44 Da). M4 and M5 had the same characteristic fragment ions as arbidol after they lose the 80 Da or the 176 Da. On the basis of these data, M4 and M5 were tentatively identified as sulfate and glucuronide conjugates of arbidol.

Using the same strategy, M6 and M7 were tentatively identified as sulfate and glucuronide conjugates of *N*-demethylarbidol (N1). M12 and M13 were tentatively identified as sulfate and glucuronide conjugates of sulfonylarbidol (N2-2). M14 and M15 were tentatively identified as sulfate and glucuronide conjugates of *N*-demethylsulfonylarbidol (M3-2).

All of these sulfate conjugates (M12 and M15) had the conspicuous in-source decomposition of $[M + H - 80]^+$ (N2-1 and M3-1) which should not be identified as metabolites.

M8–M11

M8, M9-1, and M9-2 had retention times of 23.3, 22.6, and 24.0 min with their $[M + H]^+$ values being m/z 573, 669 and 669 respectively. These were the results of m/z 493 (M1-2) plus 80 and 176 Da. The MS^2 spectrum of M8 (m/z 573)

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showed fragment ion at 448 (−125 Da) suggesting M8 to be the sulfate conjugate of sulfinylarbidol (M1-2).

The MS^3 of m/z 544 (m/z 669 → 544) produced fragment ions of m/z 529, 368, 323, and 279. There were also the results of losing CH_3 (−15 Da), SO_3 (−80 Da), SO_3 plus dimethylamine (−80 Da − 45 Da), SO_3 plus dimethylamine plus acetaldehyde (−80 Da − 45 Da − 44 Da). On the basis of these data, M9 was tentatively identified as the glucuronide conjugations of sulfinylarbidol (M1-2). More attention was paid to the in-source decomposition of $[M + H - 80]^+$ (M1-1), which had the same retention time with M8, for it could interfere with the identification of the metabolite.

For M9 at m/z 669, two LC peaks at retention times of 6.25 min (M9-1) and 6.60 min (M9-2) were observed. Same fragmentation patterns were observed for both components. The MS^2 spectrum of M9 showed fragment ions at m/z 544 (m/z 669 − 125 Da). The m/z 529 (m/z 544 − 15 Da), 368 (m/z 544 − 80 Da), 323 (m/z 544 − 80 Da − 45 Da) and 279 (m/z 544 − 80 Da − 45 Da − 44 Da) ions were detected in the MS^3 of m/z 669 → 544 as a result of losing CH_3 (−15 Da), glucuronic acid (−176 Da), dimethylamine (−45 Da) and acetaldehyde (−44 Da). The CID pathway of the M9 is deduced on the basis of the above information and is shown in Fig. 5.

Since the phenylsulfinyl moiety formed a new chirality center, the two diastereoisomers in the glucuronidation of M1 (M9-1 and M9-2) were separated in the chromatograms. Therefore, M9-1 and M9-2 might be identified as two metabolites, which were the two diastereoisomers of glucuronide conjugation for M1. Using the same strategy, M10, M11-1 and M11-2 were tentatively identified as sulfate and glucuronide conjugates of M2-2.

On the basis of the above discussion, the proposed major metabolic pathways of arbidol in humans are shown in Fig. 6.

CONCLUSIONS

The HPLC-$ITMS^n$ was used for the identification of arbidol metabolites from human urine samples. There were altogether 17 metabolites detected and identified. Major metabolites identified were glucuronide arbidol (M5) and glucuronide sulfinyl arbidol (M9-1 and M9-2).

The MS/MS behaviors of arbidol and all of its identified metabolites were studied in detail. Arbidol and its metabolites have some common fragmentation patterns as a result of the homolytic bond cleavage. This cleavage will form odd-electron ions with the loss of a radical. The arbidol fragmentation sequence is first to lose dimethylamine (45 Da), followed by the loss of acetaldehyde (44 Da), and then the phenylthio radical (109 Da). This fragmentation sequence is also observed from *N*-demethylarbidol (N1), sulfonylarbidol (N2-2), and *N*-demethylsulfonylarbidol (M3-2). However, for sulfinylarbidol (M1-2) and *N*-demethylsulfinylarbidol (M2-2), the fragmentation sequence is reversed so that the phenylsulfiny radical (125 Da) was lost first, followed by the loss of dimethylamine (45 Da), and then acetaldehyde (44 Da). The exact masses for arbidol (M0) and sulfinylarbidol (M1-2) fragment ions were determined by a Q-TOF MS.

The phase II metabolites, such as sulfate and glucuronide conjugations of arbidol (M0), *N*-demethylarbidol (N1), sulfonylarbidol (N2-2) and *N*-demethylsulfonylarbidol (M3-2) were identified by observing the neutral loss of 80 Da (SO_3) or 176 Da (glucuronic acid) from the MS^2 spectra. Sulfate and glucuronide conjugations of sulfinylarbidol (M1-2) and *N*-demethylsulfinylarbidol (M2-2) have an unusual fragmentation pattern, in which the phenylsulfinyl radical (125 Da) was first lost from the $[M + H]^+$. The loss of SO_3 group (80 Da) or glucuronic acid (176 Da) only occurs after the 125 Da losses.

Since the phenylsulfinyl moiety formed a new chirality center, the two diastereoisomer in M9 and M11 were separated in the chromatograms.

In conclusion, it was not enough to identify phase II metabolites only through the characteristic neutral loss at MS^2 spectra. Most importantly, identification of metabolites should be considered with all factors. Otherwise, it is highly possible that the metabolites identified are false positives. Good examples are the in-source fragment ions from phase II conjugates. In this case, distinct LC peaks were observed.

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