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# Pharmacokinetics and metabolite identification of a novel VEGFR-2 and Src dual inhibitor 6-chloro-2-methoxy-N-(2-methoxybenzyl) acridin-9-amine in rats by liquid chromatography tandem mass spectrometry

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ABSTRACT

A novel VEGFR-2 and Src dual inhibitor, 6-Chloro-2-methoxy-N-(2-methoxybenzyl) acridin-9-amine (MBAA), is a 9-aminoacridine derivative, but its pharmacokinetics and metabolism in body remain unknown. Using liquid chromatography tandem electrospray ionization mass spectrometry with the multiple reaction monitoring modes, we developed and validated a simple, rapid, sensitive and accurate technology for analyses of MBAA in the rat plasma, urine and bile. The micro samples were quickly prepared by 96-well plate. Chromatographic separation was performed on a $C_{18}$ column with gradient elution. High-quality linearity calibration curves were achieved over a concentration range of 1.00–3000 ng mL$^{-1}$. Intra- and inter-day precisions (RSD) were less than 8.5%, and accuracy (RE%) ranged from −2.9% to 12%. Extraction recoveries of MBAA were consistent with an average of 82.2–111.4% at three QC concentrations. When administered intravenously at a single dose of 2.0 mg kg$^{-1}$ to male SD rats, MBAA was rapidly eliminated with a $T_{1/2}$ of 0.9 ± 0.1 h and $AUC_{0-t}$ of 369 ± 44.7 ng mL$^{-1}$. We identified four direct phase I and phase II metabolites by mass difference of molecular ions between metabolites and the parent compound. Various fragmentation patterns of MBAA were used to identify and characterize its metabolites. This LC-MS/MS analysis provides a useful approach to the pharmacokinetic and metabolic study of MBAA.



## 1. Introduction

Acridine derivatives possess numerous biological activities, such as anti-inflammatory [1,2], anti-bacterial [3,4], anti-parasite [5], and anti-cancer [6]. Some of acridines, such as amsacrine [7,8] and pyrazoloacridine [9,10], have been clinically used as anticancer agents which act by intercalating with DNA and thus inhibiting DNA related enzymes, such as topoisomerase II [11] and telomerase [12,13]. Recent study indicates that the nature and position of substituents on the acridine scaffolds play a critical role in their anticancer properties. Based on a molecular docking and SVM virtual screening method [14], we have synthesized a series of 9-aminoacridine derivatives as VEGFR-2 [15,16] and Src [17] inhibitors (such as tyrosine inhibitor) to obtain acridines with better anticancer activity. Among which 6-Chloro-2-methoxy-*N*-(2-methoxybenzyl) acridin-9-amine (MBAA) [18] showed promising activity against K562 and HepG-2 cancer cells by inhibiting VEGFR-2 and Src activity, but not topoisomerase. MBAA at 10 μM significantly reduced the level of activated ERK 1/2 in a time dependant manner. Such selectivity and specificity of MBAA is beneficial to reducing host toxicity. However, the pharmacokinetic and metabolism of MBAA remain unknown.

As a novel anticancer lead compound and tyrosine kinase inhibitor with an acridine scaffold, pharmacokinetic characteristics and metabolites of MBAA *in vivo* are important parameters

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for the evaluation of its potential as an anticancer agent. Multi-analyses of the pharmacokinetics and metabolic processes *in vivo* may comprehend the mechanism of action and provide appreciable information for further structure modifications to develop more effective antitumor analogues of acridines. On the other hand, to know the process (absorption, metabolism, and elimination) of the new compound candidates in order to select the route of administration and the dose. Several methods have been reported for the quantitation of acridine derivatives, such as liquid chromatography (LC) with ultraviolet (UV) [19,20], electrochemical [21] and fluorescence detection [22]. Up to date, HPLC with different detection modes is well established, but is unable to distinguish the same kinds of mixture. Liquid chromatography tandem mass spectrometry (LC-MS/MS) with APCI source can provide the information of distinct quality [22], but uncertainty of derivatization procedures and the LOD value of APCI-MS tests limit its use as a quick biopharmaceutical analysis. Besides, in order to satisfy the requirement of sensitivity, a large volume of body fluid samples were usually necessary. To accurately know the pharmacokinetics and metabolism behavior of acridine derivatives *in vivo*, it is necessary to establish a suitable method for measuring lower concentrations of acridine derivatives in bio-samples and reducing the time and requirements for sample preparation.

In this study, an accurate, sensitive and rapid LC-ESI-MS/MS method is developed for MBAA quantification in rat plasma, urine and bile. Pharmacokinetics of MBAA was studied in rats when administered by oral or intravenous injection. Based on previous pharmacokinetic indicators of acridine derivatives [23,24], the compounds responsible for the first-pass effect in the body were supposed. The phase I/II metabolites of this novel 9-aminoacridine derivative and the fragmentation rules of metabolic products were defined.

## 2. Experimental

### 2.1. Chemicals and reagents

6-Chloro-2-methoxy-N-(2-methoxybenzyl) acridin-9-amine (MBAA) was synthesized and purified as previously described [18]. Internal standard (IS) fluoxetine and formic acid (LC-MS grade) were purchased from Sigma-Aldrich (St Louis, MO, USA). Methanol and acetonitrile (HPLC grade) were obtained from Fisher Co., Ltd. (Emerson, IA, USA). Methylcellulose, Tween-80, DMSO and other reagents of analytical grade were purchased from Fuchen Chemical Factory (Tianjin, China). A Milli-Q water system (Milford, MA, USA) was used to obtain the ultra-pure water.

### 2.2. Animals

Adult male Sprague-Dawley (SD) rats were multiply housed during acclimation and then individually housed (due to cannulations) in polycarbonate solid-bottom rat cages or metabolic cages during in-life studies that are in accordance with the Guide for the Care and Use of Laboratory Animals of the National Research Council. Animals were fed with a standard rodent diet supplied by Shanghai SLAC Laboratory Animal Co., Ltd. and water was provided ad libitum.

For absorption study, twelve rats were cannulated via carotid for blood collection. Six rats were cannulated via jugular for intravenous injection (i.v.) at 2.0 mg $kg^{-1}$ and the other six rats were orally administered at 10 mg $kg^{-1}$. All cannulations were conducted under the anesthesia of isoflurane. For the preparation of oral solution, appropriate amounts of the test particles were accurately weighed out with calibrated electrical balance into a mortar and ground with pestle. Appropriate volume of 0.5% methylcellulose with 0.1% Tween 80 was added and ground until well dispersed. The final concentration of the suspension was 1.00 mg $mL^{-1}$ and 10 mg $kg^{-1}$ per rat was delivered by gavaging. For i.v. administration, compound was dissolved in DMSO and then diluted to 2.0 mg $mL^{-1}$. One milliliter per kg per rat was administered. For metabolism and excretion studies, urine and bile were collected separately from two groups. Group I for urine ($n = 4$) was administered intravenously at a dose of 2.0 mg $kg^{-1}$, and the rats were individually housed in metabolic cages and urine was collected for 72 h in 6 batches (0–12, 12–24, 24–36, 36–48, 48–60, and 60–72 h after dosing). Group II for bile ($n = 4$) was administered i.v. at a dose of 2.0 mg $kg^{-1}$, and bile was collected by cannulating through biliary duct over 24 h period in 5 batches (0–1, 1–3, 3–6, 6–9, and 9–24 h after dosing).

### 2.3. Samples preparation

100 μL blood samples were collected into EDTA microtainers from the carotid at the indicated time points. Blood collection tubes were gently inverted at least 5 times ensuring complete mixing and immediately placed on ice. Blood was centrifuged 5 min at 5000 rpm and 4 °C to separate the plasma from the blood cells. Plasma samples were stored at −80 °C until analysis. For sample preparation, 20 μL aliquot of plasma was spiked into a 96-well plate, and 40 μL of acetonitrile containing internal standard (IS 100 nM) was added for protein precipitation. The mixture was vortexed and centrifuged at 5000 rpm for 10 min. 50 μL supernatant was mixed with 150 μL of acetonitrile in $H_2O$ (1:1, v/v) and the 10 μL resulting solution was analyzed with LC-MS/MS.

A 20 μL aliquot of urine or bile samples was spiked into a 96-well plate, and 180 μL of water containing IS was added and mixed by vortex, followed by addition of 500 μL ether. After centrifugation at 5000 rpm for 10 min, 200 μL supernatant (ether layer) was collected into a new vial and concentrated by Nitrogen Evaporators. 200 μL solvent (Acetonitrile:$H_2O$ = 1:1, v/v) was added, mixed by vortex, and centrifuged at 5000 rpm for 10 min. For urine sample, 100 μL supernatant was mixed with 100 μL of acetonitrile:$H_2O$ (1:1, v/v) and then 10 μL of the sample injected for LC-MS/MS analysis; for the analysis of bile, 50 μL supernatant was mixed with 150 μL of acetonitrile:$H_2O$ (1:1, v/v) and 10 μL of the sample injected into LC-MS/MS assay.

### 2.4. LC-MS/MS conditions

Chromatographic separations were accomplished with Waters Alliance 2695 HPLC system (Waters, USA). Detection was performed on a Quattro Premier XE Mass Spectrometer (Waters, UK) equipped with an Electron Spray Ionization (ESI) positive ion mode. All mass spectral data were acquired in the centroid mode. Data acquisition and processing were obtained using Masslynx 4.1 Analyst Software Quanlynx (Waters, USA).

Quantification of MBAA in plasma, urine and bile: Samples were separated using a Waters X-Bridge $C_{18}$ column (100 mm × 4.6 mm ID, 5 μm) at room temperature. Flow rate was maintained at 1.0 mL $min^{-1}$. Mobile phases were used A ($H_2O$ with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid). The gradients of the mobile phases were listed in Supplemental Table S1. MS conditions were as follow: source temperature was 200 °C, desolvation temperature was 350 °C, gas desolvation was set at 1000 L $h^{-1}$ and capillary voltage was 3.2 kV. Under the multiple reaction monitoring (MRM) mode, precursor → product transition pair was mass-to-charge ratio $m/z$ 379.1 → 121.3 (cone voltages 40 V, collision energy 20 V) for MBAA, and $m/z$ 310.1 → 44.0 (cone voltages 20 V, collision energy 15 V) for IS. Cone gas was set at 20 L $h^{-1}$. Dwell time was 0.1 s.

Metabolite identification of MBAA in urine and bile samples: separations were achieved on a Waters X-Bridge $C_{18}$ column (100 mm × 2.1 mm ID, 3.5 μm). Flow rate was maintained at 0.3 mL min$^{-1}$. The mobile phases were A ($H_2O$ with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid) with a gradient in Supplemental Table S1. Mass spectral data was obtained in electrospray ionization with positive ion mode. Conditions were as follows: capillary voltage 3.2 kV, cone voltage 35 V, source temperature at 200 °C, desolvation temperature at 350 °C, and gas desolvation at 600 L h$^{-1}$. Data were collected in the centroid mode between $m/z$ 100 and 1000.

### 2.5. Validation of the methods

Blank plasma, urine and bile samples were also collected from rats dosed with vehicle. Nominal standards were curved from 1.00 to 3000 ng mL$^{-1}$. The concentrations of nominal standards were 1.00, 2.00, 10.0, 50.0, 100, 500, 1000 and 3000 ng mL$^{-1}$. Calibration curves of MBAA were constructed by plotting the peak-area ratios of the target compound/IS versus concentrations of the compound in plasma, urine and bile, respectively. Linearity was determined using a linear least-squares regression. The response of the compound at the concentration of the low limit of quantification (LLOQ) were produced a signal-to-noise ratio (S/N) at 5. The chromatogram of each blank biological fluid sample was compared with that of the corresponding plasma, urine and bile sample with the target compound and IS.

Accuracy and precision of the method was validated by measuring quality control (QC) samples at three concentration levels (3.00, 800 and 2700 ng mL$^{-1}$) in six replicates on three validation days. The assay accuracy of three fluid samples was expressed as RE%. Intra- and inter-day precision was expressed as relative standard deviation (RSD). Extraction recovery of MBAA and IS in the different biological fluids was evaluated by standards with three QC levels ($n$ = 6).

Stability of MBAA in rat plasma, urine and bile was assessed by analyzing five replicates of QC samples ($n$ = 5) at three concentrations of 3.00, 800 and 2700 ng mL$^{-1}$. The stability of 4 h was performed by the QC samples during analysis at room temperature. Long-term stability was tested after storage of samples at −80 °C for two months. The freeze/thaw stability was examined at three freeze/thaw cycles between −80 °C and room temperature. The final reconstituted sample stability was assessed by left QC samples in the HPLC auto-sampler for 24 h at 4 °C.

### 2.6. Pharmacokinetic studies

Blood samples (0.1 mL each) were collected into EDTA-treated microtainers from the carotid at the following time points: 0.083, 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h after MBAA administration. Urine was collected over a 72 h period in 6 batches (0–12, 12–24, 24–36, 36–48, 48–60 and 60–72 h after dosing). Bile was collected over 24 h period in 5 batches (0–1, 1–3, 3–6, 6–9 and 9–24 h after dosing). The volume of both urine and bile samples during each time interval was measured and recorded.

A non-compartmental pharmacokinetic analysis (WinNonlin 5.2, Pharsight Corp., Mountain View, CA, USA) [25] was performed on the plasma concentrations for each rat. Parameters estimated included $AUC_{0-t}$ (the area under the plasma concentration versus time curve from time 0 to the last measurable concentration calculated using the linear trapezoidal rule), the $AUC_{0-\infty}$ (calculated as $AUC_{0-\infty} = AUC_{0-t} + C_t/\lambda_z$, where $C_t$ is the last quantifiable concentration at time $t$ and $\lambda_z$ is obtained by log linear regression using the terminal points of the plasma concentration–time curve), $T_{1/2}$ (the elimination half-life, calculated as 0.693/$\lambda_z$), CL (the total body clearance, calculated as dose/$AUC_{0-\infty}$), and $V_z$ (the volume of distribution, calculated by CL/$\lambda_z$).

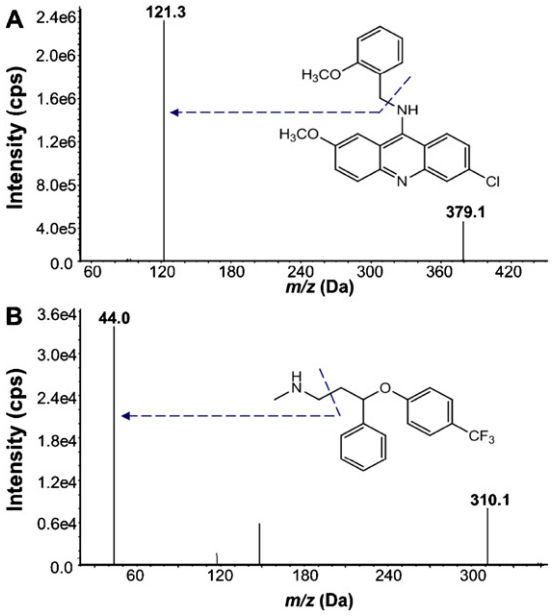


**Fig. 1.** Product ion mass spectra of $[M+H]^+$. (A): MBAA (MW = 378.1 Da) and (B): fluoxetine (IS, MW = 309.1 Da).

### 2.7. Metabolite identification

The metabolites of MBAA in rat urine and bile were identified by a sensitive LC-ESI-MS/MS method. For MS/MS analysis to identify metabolites, the collision energies were set at 5–30 V. The molecular structures of metabolites are taken on the basis of the characteristics of their parent ions, product ions and chromatographic retention time.

## 3. Results and discussion

### 3.1. LC-MS/MS

For the quantitation of MBAA, the mobile phase was optimized. Gradient elution was used for narrowing the peaks of MBAA and IS and shortening running time of the chromatography. The running time for each sample was just for 2.5 min. ESI was chosen as the interface based on the structure of compounds which made an intense signal. Sensitivity ratio of the MRM scan mode was significantly superior to that of the selected ion recording (SIR). Cone voltage and collision energy were optimized for each analyte by infusion of 500 ng mL$^{-1}$ of standard solutions with the flow rate of 10 μL min$^{-1}$ via a syringe pump. The full-scan positive ion scans of MBAA and IS showed ionization molecular ions ($[M+H]^+$) of $m/z$ 379.1 and $m/z$ 310.1, respectively. Fragments in the collision cell, the most abundant and stable product ions (Fig. 1), were at $m/z$ 121.3 for MBAA and at $m/z$ 44.0 for IS, respectively. 0.1 s dwell time and 0.02 s spacing time was set for achieving favorable chromatographic peak statistics (over 12 points per peak).

For the identification of metabolites, vehicle and MBAA-dosed bile and urine samples were separated and detected by changing the chromatographic conditions. The optimal LC-MS/MS conditions were described in Section 2.4. Based on the full scan, several peaks were unique to the dosed samples compared to vehicle control (Fig. S1). These unique peaks present in the MBAA-dosed samples were considered metabolites of MBAA. The collision energy at 5 V, 15 V, and 30 V were used for MS/MS scan of the unique peaks,

**Table 1**
Accuracy, intra- and inter-day precision and recovery of MBAA in plasma, urine and bile samples ($n = 6$).

| Samples | Concentration ($ng\,mL^{-1}$) | Intra-day | | Inter-day | | Extraction recovery | |
|---|---|---|---|---|---|---|---|
| | | RE (%) | RSD (%) | RE (%) | RSD (%) | Mean (%) | RSD (%) |
| Plasma | 3.00 | 8.3 | 7.7 | 12 | 7.4 | 111.4 | 5.0 |
| | 800 | 0.59 | 4.9 | −0.18 | 2.9 | 96.4 | 4.9 |
| | 2700 | 1.8 | 1.7 | −1.2 | 1.8 | 99.6 | 0.7 |
| Urine | 3.00 | 3.9 | 4.1 | 2.6 | 5.0 | 96.0 | 4.3 |
| | 800 | −0.30 | 2.1 | −2.9 | 2.9 | 90.5 | 7.5 |
| | 2700 | −1.0 | 5.9 | 0.3 | 3.7 | 93.4 | 5.3 |
| Bile | 3.00 | −0.9 | 7.3 | 5.0 | 8.5 | 91.2 | 8.7 |
| | 800 | 3.9 | 4.3 | 0.59 | 4.4 | 82.2 | 6.8 |
| | 2700 | 5.3 | 6.3 | −1.0 | 7.5 | 83.5 | 2.6 |

respectively. The structure of these peaks were identified by several messages given by MS/MS spectra that were performed to carefully study for taking possible structures into consideration (see Section 3.3).

### 3.2. Method validation

Fig. 2 shows the typical LC-MS/MS chromatograms of blank plasma (A), urine (B), and bile (C). In Fig. 2, (a) indicates blank sample, (b) denotes blank sample spiked with MBAA at LLOQ concentration of $1.00\,ng\,mL^{-1}$, and (c) represents the sample from a rat dosed (i.v.) at $2.0\,mg\,kg^{-1}$ of MBAA. Retention times were stable. No interference peaks were observed at eluting positions of MBAA and IS in three repeat analysis. The retention time was at 1.82 min for MBAA and 1.79 min for IS.

For method validations, calibration standards were prepared and analyzed during a period of three days. The calibration curves of MBAA in plasma, urine and bile were linear in the range of 1.00–3000 $ng\,mL^{-1}$. The regression equation of plasma, urine and bile standard curves was: $y = 0.00209\,x + 0.00139$, $y = 0.00128\,x + 0.000889$, and $y = 0.00177\,x + 0.000561$, respectively, where $y$ is the peak area ratio of MBAA to IS and $x$ is the sample concentration of MBAA. All correlation coefficients were greater than 0.995. The lowest limit of detection (LLOD) at a signal-to-noise ratio of 5:1 was estimated at $1.00\,ng\,mL^{-1}$.

The accuracy and precision data for intra-day and inter-day analyses are shown in Table 1. The results showed that this LC-MS/MS method had satisfactory reproducibility with excellent accuracy (RE%) ranging from −2.9% to 12% and precision (RSD) less than 8.5% within three QC samples. Extraction recoveries of MBAA were ranged from 82.2% to 111.4% at three QC concentrations (Table 1).

Stability study indicated no significant changes in the concentration of MBAA in three QC samples ($n = 5$) for a short-term (4 h) at

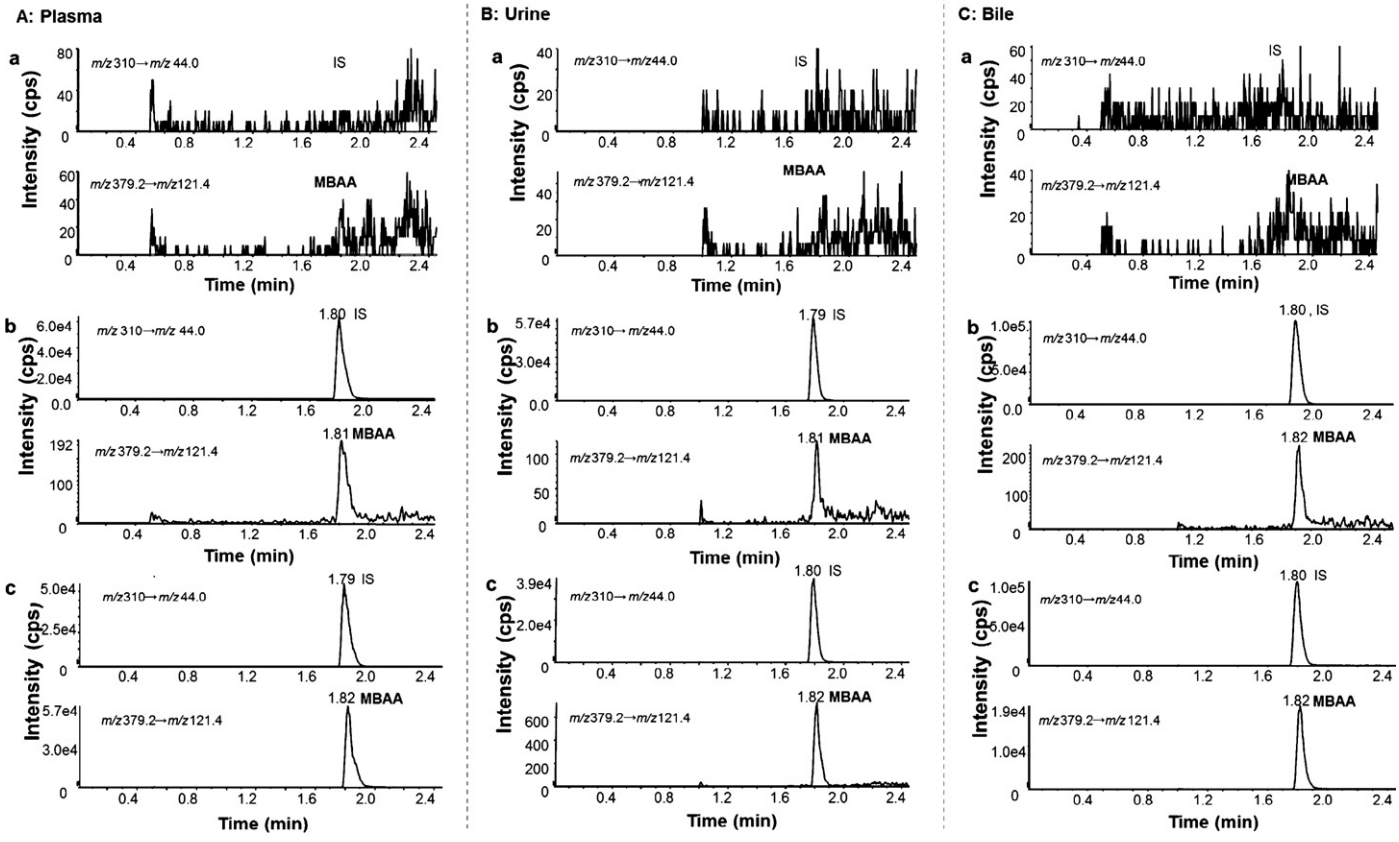


**Fig. 2.** MRM chromatograms of MBAA in body fluid samples. (A): Plasma, (B): urine and (C): bile. In the sample, (a), (b) and (c) represent blank, blank samples spiked with a standard at $1.0\,ng\,mL^{-1}$ and samples collected at 5 min following i.v. administration, respectively.

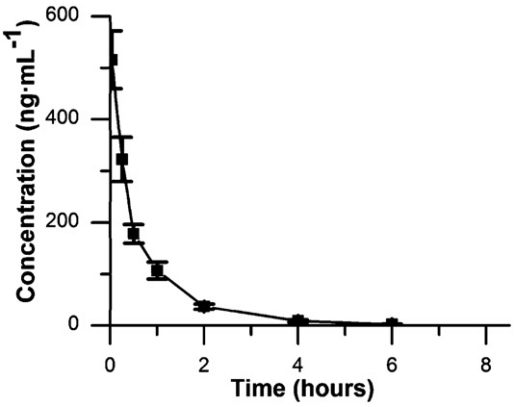


**Fig. 3.** Mean plasma concentration–time curve of MBAA administrated i.v. at 2.0 mg kg$^{-1}$ to male SD rats. Values indicate mean ± SD from six rats.

room temperature and long-term (two months) at −80 °C, with the freeze and thaw cycles, or in auto-sampler at 4 °C for 24 h. Stability tests (RE%) were between −7.6% and 12% of initial concentration. All tests were repeated at least five times and RSD was less than 15% (Supplemental Table S2).

### 3.3. Pharmacokinetics of MBAA

The validated LC-MS/MS method was successfully applied to pharmacokinetic studies of MBAA. The plasma clearance of MBAA administrated i.v. at 2.0 mg kg$^{-1}$ is illustrated in Fig. 3. Supplemental Table S3 summarizes the details in each rat. Plasma MBAA was low to a level close to the LLOQ (1 ng mL$^{-1}$) in oral administration of 10 mg kg$^{-1}$ (Supplemental Table S4), indicating poor oral bioavailability of this compound. Estimated pharmacokinetic parameters of intravenous MBAA in rats, such as $T_{1/2}$, $AUC_{0-t}$, $AUC_{0-\infty}$, $V_z$, CL and MRT are summarized in Table 2. Data showed that plasma MBAA was rapidly cleared up with an average $T_{1/2}$ at 0.9 ± 0.1 h, and MBAA was undetectable 8 h after intravenous administration. The reasons for poor oral bioavailability of MBAA are currently unknown, but most likely are due to the strong first-pass clearance by the liver of SD rats as indicated by the short $T_{1/2}$ in intravenous administrations. Further study is warranted.

Secretion of intact MBAA was further investigated. As shown in Fig. 4, MBAA was secreted at less than 0.010% by urine or 0.008% by bile, after administrated i.v. at 2 mg/kg, suggesting that MBAA is mainly cleared by metabolism.

### 3.4. MBAA metabolites in rat bile and urine

MBAA-dosed bile samples were analyzed in parallel with blank controls under the chromatographic conditions described in Section 2.4. The obtained selective ion monitoring (SIM) scan chromatograms were shown in Fig. S1. The peaks uniquely present in MBAA-dosed bile were designated as M0 ($m/z$ 379.1), M1 ($m/z$ 365.0), M2 ($m/z$ 527.2), M3 ($m/z$ 541.3), M4 ($m/z$ 557.0), with a retention time of 13.83, 13.15, 11.59, 12.39, and 12.32 min, respectively. The SIM chromatograms are shown in Fig. S2. The retention time and mass data of the parent and fragment ions for MBAA and its metabolites are list in Table 3.

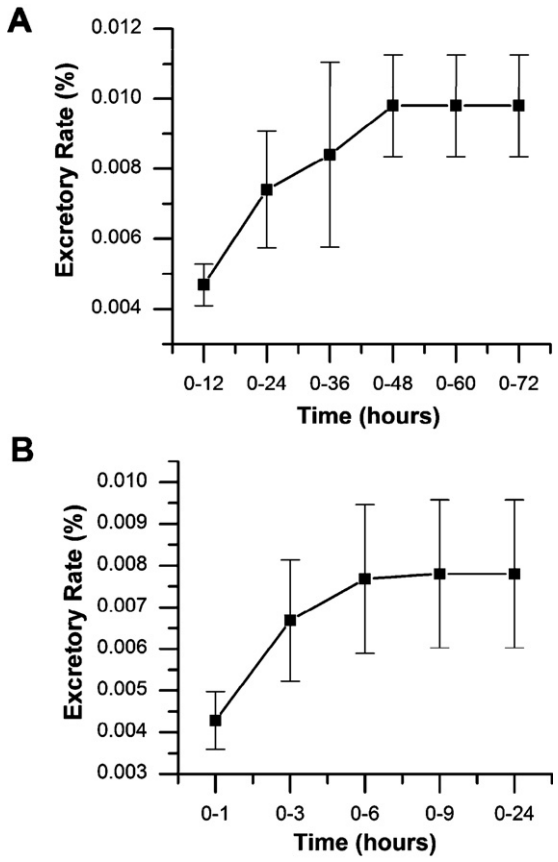


**Fig. 4.** Cumulative percentage of MBAA excreted in urine (A) and bile (B). Data represents mean ± SD from four rats.

M1, eluted at 13.15 min, showed a molecular ion $[M+H]^+$ at $m/z$ 365.0 that was a metabolite with a methyl less than the parental MBAA (M0). This may be a demethylated product of MBAA. The major fragment ion at $m/z$ 245.2 was a benzyl group loss from the molecular ion. The fragment ion at $m/z$ 121.3 was indicative of benzyl with methoxy part (Fig. 5A). The M1 is proposed to be an O-demethyl metabolite of MBAA.

M2, present at 11.59 min, was characterized with the molecular ion $[M+H]^+$ at $m/z$ 527.2. Its major fragment was at $m/z$ 351.1, derived from a loss of glucuronic acid (176 Da, $C_6H_8O_6$) of the parental ion. The fragment ion at $m/z$ 245.0 and $m/z$ 107.0 was shown in Fig. 5B. Therefore, M2 is suggested as O-di-demethyl and mono-O-glucuronide conjugated metabolite of MBAA. Due to the insufficient fragmentation information yet, the conjugation position could not be determined currently.

**Table 2**
Main pharmacokinetic parameters (mean ± SD) of MBAA (2.0 mg kg$^{-1}$) following intravenous administration in rat plasma ($n$ = 6).

| Parameters | Mean ± SD |
|---|---|
| $T_{1/2}$ (h) | 0.9 ± 0.1 |
| $C_0$ (ng mL$^{-1}$) | 554 ± 61.9 |
| $AUC_{0-t}$ (ng h mL$^{-1}$) | 369 ± 44.7 |
| $AUC_{0-\infty}$ (ng h mL$^{-1}$) | 372 ± 45.1 |
| $V_z$ (L kg$^{-1}$) | 7.45 ± 1.53 |
| CL (mL min$^{-1}$ kg$^{-1}$) | 91 ± 12.3 |
| MRT (h) | 0.9 ± 0.03 |
| $V_{ss}$ (L kg$^{-1}$) | 5.01 ± 0.630 |

**Table 3**
Chromatographic retention time and mass spectrometric data of MBAA and metabolites in rats.

| Compound | Parent ion ($m/z$) | $T_R$ (min) | Fragment ion ($m/z$) |
|---|---|---|---|
| M0 | 379.1 | 13.83 | 121.3 |
| M1 | 365.0 | 13.15 | 121.0, 245.2 |
| M2 | 527.2 | 11.59 | 107.0, 245.0, 351.1 |
| M3 | 541.3 | 12.39 | 121.0, 245.2, 365.0 |
| M4 | 557.0 | 12.32 | 123.0, 259.1, 381.0 |

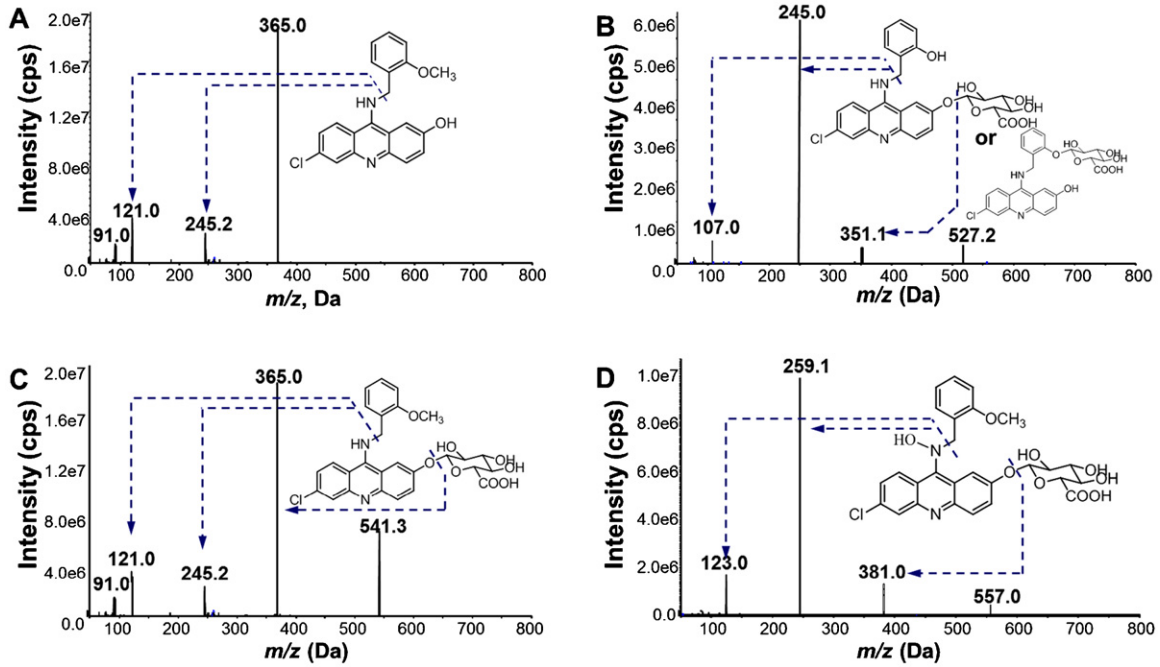


**Fig. 5.** MS/MS mass spectrum of MBAA metabolites. (A) M1 ($m/z$ 365.0, $T_R$ = 13.15 min) in bile, (B) M2 ($m/z$ 527.2, $T_R$ = 11.59 min), (C) M3 ($m/z$ 541.3, $T_R$ = 12.39 min) and (D) M4 ($m/z$ 557.0, $T_R$ = 12.32 min).

M3 at 12.39 min denoted a molecule ion at $m/z$ 541.3 which was presumed to be a glucuronidated adduct ion because it produced a deprotonated molecular ion at $m/z$ 365.0. The major fragment ions were similar to M1 at $m/z$ 245.2 and $m/z$ 121.3 (Fig. 5C). Therefore, M3 is an *O*-glucuronide conjugate metabolite of M1.

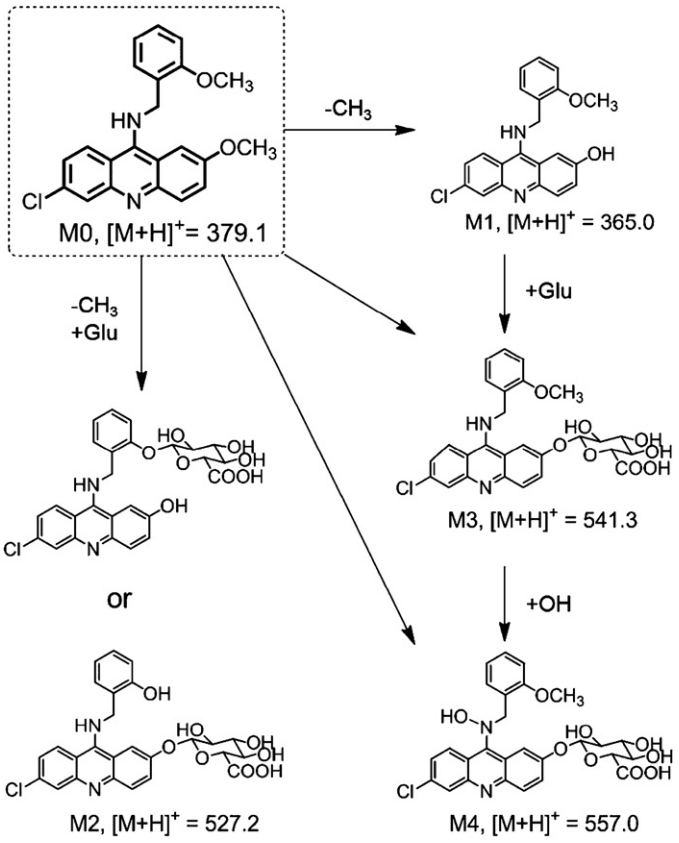


**Fig. 6.** Hypothetic metabolic pathways of MBAA in rats.

M4 had a retention time of 12.32 min and a molecular ion $[M+H]^+$ at $m/z$ 557.0. This metabolite was an oxygen atom more than the M3, suggesting a hydroxylation product of M3. The major fragment ion at $m/z$ 381.1 was a loss of glucuronic acid from the M4 and an oxygen atom more than the fragment ion of the M3. The fragment ions at $m/z$ 123.0 and $m/z$ 259.1 were the two product ions of rearrangement reactions of the parental compound (M4), suggesting that the hydroxylation position was not on the benzyl moiety, but most likely on the nitrogen of parhelium structure (Fig. 5D). M4 is the hydroxyl metabolite of M3.

As shown in Fig. 5B–D, the results of neutral loss scan of 176 further confirmed the structure of a glucuronide conjugate for metabolites M2, M3 and M4. Fig. 6 summarizes the proposed metabolic pathways of MBAA in rats. Of note, these metabolites were hardly detected in the urine under the same full scan conditions, suggesting that MBAA was mainly metabolized in the liver and subsequently, the metabolites were secreted into bile, but into circulation blood for kidney excretion.

The short $T_{1/2}$ and multiple metabolites in bile of MBAA suggest a strong first-pass clearance in the liver, which endorses our finding of the extremely low oral bioavailability of MBAA in rats. This data enlightens the novel acridine compound design in the future. Structural modifications may be necessary to increase the rate of first-pass.

## 4. Conclusions

This work developed and validated a specific LC-MS/MS method for the quantitation of 6-Chloro-2-methoxy-N-(2-methoxybenzyl) acridin-9-amine and identification of its metabolites in the rats' body fluid samples. This method showed excellent sensitivity, linearity, precision and accuracy. It was successfully applied to evaluation of pharmacokinetics of MBAA in rats. A LC-MS/MS based systematic approach for rapid metabolite identification of compounds such as MBAA that undergo multiple metabolic pathways from biological matrix is appreciable for dissection of pathways of phase I and II metabolism. This present study also describes the pharmacokinetics of MBAA in detail, providing informative data to

design and structural modifications of novel acridine derivatives. In addition, this study also presents a feasible and generally applicable strategy for the complicated metabolite identification of acridine analogues.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2011.11.061.

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