Journal of Pharmaceutical and Biomedical Analysis 55 (2011) 288–293

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Validated LC–MS/MS method for the determination of maackiain and its sulfate and glucuronide in blood: Application to pharmacokinetic and disposition studies

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ARTICLE INFO

Article history:
Received 12 October 2010
Received in revised form 15 January 2011
Accepted 18 January 2011
Available online 22 January 2011

Keywords:
Maackiain
Maackiain-7-glucuronide
Maackiain-7-sulfate
Pharmacokinetics
UPLC–MS/MS

ABSTRACT

The purpose of this study was to develop a simultaneous, sensitive and reproducible UPLC–MS/MS method to quantify maackiain and its phase II metabolites, maackiain-sulfate (M-7-S) and maackiain-glucuronide (M-7-G). A Waters BEH C_{18} column was used with acetonitrile/water as mobile phases. Analysis was performed under negative ionization electrospray mass spectrometer via the multiple reaction monitoring (MRM). The one-step protein precipitation by methanol was used to extract the analytes from plasma. The results showed that the linear response range was 5000–9.75 nM for maackiain, M-7-S, and M-7-G. The lower limit of detection (LLOD) was 4.88 nM for these three analytes. The intra-day variance is less than 12.4% and accuracy is in 85.7–102.0%. The inter-day variance is less than 11.2% and accuracy is in 89.6–122.2%. The analysis was done within 4.0 min. Only 20 μl of blood is needed for the analysis due to the high sensitivity of this method. The validated method was used for pharmacokinetic study in A/J mouse, maackiain Caco-2 cell culture model experiment, and maackiain glucuronidation/sulfation metabolism studies. The applications revealed that this method can be used for maackiain, M-7-S, and M-7-G analysis in both bioequivalent buffer and in blood.

1. Introduction

Maackiain (Fig. 1), a flavonoid analog, was originally isolated from *Maackia amurensis* var [1], and widely distributed in different plant genus (e.g. *Maackia* [2], *Sophora* [3,4], *Trifolium* [5], *Millettia* [6], *Caragana* [7]) of Fabaceae family. Maackiain has been proven to posses multiple bioactivities *in vitro* including antimicrobial effects [3], anticancer effects via cellular toxicity, induction of apoptosis [8,9], and inhibition of aryl hydrocarbon hydroxylase [10]. Varieties of research efforts have been reported on this compound including total chemical synthesis [11], biotransformation by fungi or plant cell culture [12,13], and plant genetics [14]. During our lung cancer chemoprevention with anti-tumor B (ATB) investigation, maackiain was isolated as a pure compound and was proven to possess 10 times higher anti-proliferation activity than that of ATB against lung cancer LM1 cell lines in MTT assay (data not shown). To further demonstrate the *in vivo* activity of ATB, more disposition and pharmacokinetic studies of maackiain are of significant value. However, to our knowledge, the analysis method for this compound has not been developed.

Flavonoids have been shown to possess multiple activities *in vitro*, but their *in vivo* activities remain uncertain, perhaps because of their poor bioavailability [15]. Phase II metabolism is the major barrier to flavonoid bioavailability. Maackiain is expected to have similar pharmacokinetic and dispositional characteristics to flavonoids due to its structural similarity to flavonoids. However, phase II metabolism of this compound, either *in vitro* or *in vivo*, has not been reported.

Therefore, the purpose of this research is to establish a sensitive and reproducible UPLC–MS/MS method that can be used to quantify maackiain and maackiain-glucuronide/sulfate simultaneously in both plasma and biological samples and to apply this method for further *in vitro*/*in vivo* disposition and pharmacokinetic studies.

2. Experimental

2.1. Chemicals and regents

Maackiain was bought from Ruicong Ltd. (Shanghai, China). Formononetin was purchased from LC Laboratory (Woburn, MA). Uridine-5′-diphosphate-β, D-glucuronic acid ester (UDPGA), 3′-phosphoadenosine-5′-phosphosulfate (PAPS), D-saccharic-1,4-lactone monohydrate, magnesium chloride, and Hanks' balanced salt solution (powder form) were purchased from Sigma–Aldrich (St. Louis, MO). All other materials (typically analytical grade or better) were used as received.

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doi:10.1016/j.jpba.2011.01.015

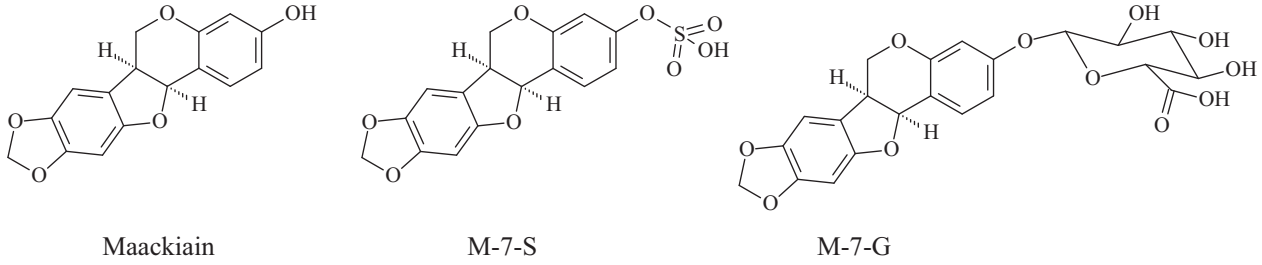

Fig. 1. Chemical structures of maackiain, M-7-S, and M-7-G.

2.2. Instruments and conditions

2.2.1. UPLC

UPLC conditions for analyzing maackiain and maackiain conjugates were: system, Waters Acquity™ with diode array detector (DAD); column, BEH C_{18} column (50 mm × 2.1 mm I.D., 1.7 μm, Waters, Milford, MA, USA); mobile phase A (MPA), 100% water; mobile phase B (MPB), 100% acetonitrile; gradient, 0–0.5 min, 0–10% MPB, 0.5–2.5 min, 10–50% MPB, 2.5–3.0 min, 50–75% MPB, 3.0–3.5 min, 75–95% MPB, 3.5–3.7 min, 95–0% MPB, 3.7–4.0 min, 0% MPB; flow rate, 0.6 ml/min; column temperature, 60 °C; injection volume, 10 μl. Formononetin (1 μM in methanol) was used as the internal standard.

2.2.2. UPLC–MS

The UPLC–MS analysis was performed on an API 3200 Qtrap triple quadrupole mass spectrometer (Applied Biosystem/MDS SCIEX, Foster City, CA, USA) equipped with a TurboIonSpray™ source. The structures of maackiain metabolites in plasma were established through UPLC–MS/MS method and the concentration of maackiain, maackiain metabolites were determined by using MRM (Multiple Reaction Monitoring) method. The instrument dependent parameters for mass spectrum were set as follows: ionspray voltage, −4.5 kV; ion source temperature, 600 °C; nebulizer gas (gas 1), nitrogen, 50 psi; turbo gas (gas 2), nitrogen 50 psi; curtain gas, nitrogen 10 psi. Unit mass resolution was set in both mass-resolving quadruples Q1 and Q3. Compound-dependent parameters were listed in Table 1.

2.3. Biosynthesis of M-7-G, and M-7-S

2.3.1. Mouse liver S9 fraction preparation

Male mouse liver S9 fraction was prepared from A/J mice according to the reported procedures and stored at −80 °C until use [16]. The animal protocols used in this study were approved by the University of Houston's Institutional Animal Care and Uses Committee.

2.3.2. Phase II metabolism reaction systems

The glucuronidation and sulfation procedures have been reported previously from our lab [17,18]. Genistein was used as a positive control. Briefly, 10 μM maackiain (200 μl) was incubated with liver S9 fraction (final concentration = 0.0027 mg protein/ml) at 37 °C and I.S. (50 μl of 1.0 μM formononetin in methanol) was added to terminate the reaction. The samples were vortexed for 30 s, and centrifuged at 15,000 rpm for 10 min prior to UPLC–MS/MS analysis. The reaction was monitored every hour by UPLC–MS/MS until maackiain was not detectable (4 h). Another 10 μM maackiain was incubated 4 h to produce M-7-G. The crude solution will be used as M-7-G stock solution and its final concentration was determined by a maackiain standard curve and a conversion factor of the relevant extinction coefficients (Section 2.4).

For sulfation, the procedure also followed Refs. [17,18]. The 10 μM maackiain was incubated with S9 fraction for 4 h to produce M-7-S. The crude solution is used as M-7-S stock solution and its final concentration was determined using a maackiain standard curve and a conversion factor of the relevant extinction coefficients (Section 2.4).

2.4. Determination of conversion factors

Determination of conversion factors (K) has been described previously [19,20]. Briefly, a maackiain standard curve was prepared in the same solution as described in Section 2.3.2 but without S9 fraction, which was replaced with water. The conversion factors were determined by comparing the UV absorption peak area of maackiain and those of M-7-G and M-7-S at 310 nm with following its 100% conversion to after a complete glucuronide or sulfate (Eq. (1)). Three different concentrations (5, 10, 20 μM) were performed. The extinction coefficient effect (EC) was the average of EC values these three concentrations.

$$K = \frac{PA_c}{PA_m} \quad (1)$$

whereas PA_c is peak area of conjugates (M-7-G and M-7-S) and PA_m stands for peak area of maackiain.

2.5. Method validation

2.5.1. Calibration curve and LLOD

Calibration curves were prepared in the same way as described in Section 2.6.4. The linearity of each calibration curve was determined by plotting the peak area ratio of maackiain, M-7-G, and M-7-S to I.S. Least-squares linear regression method ($1/x^2$ weight) was used to determine the slope, intercept and correlation coefficient of linear regression equation. The lower limit of detection (LLOD) was defined based on a signal-to-noise ratio of 10:1.

2.5.2. Precision and accuracy

The "intra-day" and "inter-day" precision and accuracy of the method were determined with QC samples at three different concentrations on the same day or on three different days.

2.5.3. Extraction recovery and matrix effect

The extraction recoveries of maackiain, M-7-G, and M-7-S were determined by comparing the relative peak areas obtained from blank plasma spiked with analytes and those obtained from water spiked with the same amount of analytes. Matrix effect was determined by comparing the peak areas of blank plasma extracts spikes with analytes and I.S. with those of the standard solutions dried and reconstituted with a mobile phase. These evaluations were performed according to the recommended validation procedures reported by Matuszewski et al. [21].

2.5.4. Stability

Short-term (25 °C for 4 h), long-term (−80 °C for 7 days) and three freeze–thaw cycle stabilities were determined.

Table 1
Compound-dependent parameters in UPLC–MS analysis.

Compound	Q1 (*m*/*z*)	Q3 (*m*/*z*)	DP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)	Dwell time (ms)
Maackiain	283.0	254.0	−50	−10	−26	−17	−2	100
M-7-S	363.0	283.0	−50	−10	−26	−30	−2	100
M-7-G	459.1	113.2	−18	−10	−22	−22	−1	100
Formononetin	267.1	252.1	−47	−10	−23	−26	−2	100

2.6. Pharmacokinetic study

2.6.1. Animals

A/J mice (male, 20–25 g, 8–10 weeks old) were from Harlan Laboratory (Indianapolis, IN) and kept in an environmentally controlled room (temperature: 25 ± 2 °C, humidity: 50 ± 5%, 12 h dark-light cycle) for at least 1 week before the experiments. The mice were fasted overnight before the day of the experiment.

2.6.2. Experimental design

The animal protocols used in this study were approved by the University of Houston's Institutional Animal Care and Uses Committee. Mice were fasted for 12 h with free access to water prior to the pharmacokinetic study. The drug was administrated at dose of 2.5 mg/kg in saline/alcohol (60:40). Briefly, after a mouse was anesthetized with isoflurane gas, maackiain was injected from tail vein. Afterwards, mouse tail was snipped near the tip of the tail to allow the collection of blood samples. Blood samples (about 25 μl) were collected in heparinized tubes at 5, 15, 30, 60, 90, 120, 240, 360, 480, and 1440 min, respectively. The blood samples were stored at −80 °C until analysis.

2.6.3. Plasma sample preparation

The blood sample (20 μl) was spiked with 20 μl of 50 mM potassium phosphate buffer (KPI, pH = 7.4) and 80 μl of I.S. (formononetin in methanol, 1 μM). The mixture was vortexed for 1 min. After centrifugation at 15,000 rpm for 15 min, the supernatant was transferred to a new tube and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 80 μl of 50% methanol aqueous solution and centrifuged at 15,000 rpm for 15 min. 10 μl of supernatant was injected into the UPLC–MS/MS system for quantitative analysis.

2.6.4. Preparation of standard and quality control samples

Calibration standard samples were prepared in KPI by diluting maackiain, M-7-G, and M-7-S stock solution to final concentration of 5000.0, 2500.0, 1250.0, 625.0, 312.5, 156.0, 78.0, 39.0, 19.5, 9.75, 4.88, and 2.44 nM, respectively. The calibration standard samples were prepared by spiking the blank mouse blood (20 μl) with above samples in KPI (20 μl) and 80 μl of I.S. The quality control (QC) samples for each compound were prepared at three different concentrations in the same way as the blood samples for calibration were prepared. The QC samples were stored at −80 °C until analysis.

2.6.5. Pharmacokinetic parameter calculation

Maackiain and its metabolites' pharmacokinetic parameters were calculated by the non-compartmental method, using WinNonlin 3.3 (Pharsight Corporation, Mountain View, California).

2.7. Caco-2 cell culture model study

2.7.1. Cell culture

Cell culture was prepared according to previous study in our lab [18,22]. Cells were used at passages 41–49.

2.7.2. Transport experiments in the Caco-2 cell culture model

The experiment protocol and calculation were described in our previous reports [18,22]. Briefly, maackiain solution (10 μM) was loaded onto the apical or basolateral (donor) side. Five donor samples (500 μl) and five receiver samples (500 μl) were taken at 0, 1, 2, 3, and 4 h followed by the addition of 500 μl of fresh donor solution to the donor side or 500 μl of fresh buffer to the receiver side. The samples were then analyzed by UPLC–MS/MS.

The apparent permeability coefficient (*P*) was determined by the equation

$$P = \frac{(dQ/dt)}{A \times C_0}$$

where dQ/dt is the drug permeation rate (μmol/s), A is the surface area of the epithelium (cm^2), and C_0 is the initial concentration in the donor compartment at time 0 (mM).

2.8. Maackiain phase II metabolism rate

The maackiain glucuronidation and sulfation rates were determined by measuring the amount of metabolites formed in A/J mouse S9 fraction reaction divided by reaction time (75 min) and protein concentration of S9 fraction (final concentration = 0.0027 mg protein/ml).

3. Results and discussion

3.1. Analysis method optimization

The method was set up by optimizing UPLC and MS/MS condition to obtain the best possible sensitivity. Methanol, acetonitrile, 2.5 mM ammonium acetate (pH = 7.6), 0.1% formic acid (pH = 2.5), and 100% water were tested as potential mobile phases. The negative ionization of maackiain in the instrument was the best with acetonitrile and water as the mobile phases. A gradient elution (Section 2.2.1) was used to avoid "cross-talk" peaks. In order to obtain a sharp and symmetrical peak, the column temperature was set up at 60 °C and the flow rate was 0.6 ml/min.

For the MS condition, both positive and negative scan modes were tested and the negative mode was selected due to higher sensitivity. MRM scan type was used to monitor both pseudo-molecular and fragments ions, which make the method more specific. The compounds and instrument dependent parameters were optimized by tuning these three analytes separately. The results are shown in Table 1.

3.2. Method validation

3.2.1. Specificity, linearity and sensitivity

The method validation was conducted in blank mouse blood. The standard curves were linear in the concentration range of 5000–9.75 nM for maackiain, M-7-G, and M-7-S. The lower limit of detection (LLOD) was 4.88 nM for these three analytes.

3.2.2. Accuracy and precision

Accuracy, intra-day and inter-day precision were determined by measuring six replicates of QC samples at three concentration

Table 2
Intra-day and inter-day precision in the analysis of maackiain, M-7-G, and M-7-S.

Analyte	Linear range (nM)	UPLC–MS/MS				
		Concentration (nM)	Intra day		Inter day	
			Accuracy (bias, %)	Precision (CV, %)	Accuracy (bias, %)	Precision (CV, %)
Maackiain	5000–9.75	2500	85.7	8.4	89.6	4.5
		312.5	96.4	8.2	96.4	8.9
		39.0	99.8	4.1	104.3	4.2
M-S	5000–9.75	2500	102.0	6.1	107.4	4.5
		312.5	97.7	10.8	107.2	9.2
		39.0	100.7	9.5	104.3	9.7
M-G	5000–9.75	2500	97.9	9.9	112.2	4.7
		312.5	98.2	8.09	89.9	7.4
		39.0	96.2	12.4	102.5	11.2

Table 3
Recovery of maackiain, M-7-G, and M-7-S at high, medium, and low concentrations.

C (nM)	Maackiain		M-7-S		M-7-G	
	Mean	SD	Mean	SD	Mean	SD
39	79.11	0.01	84.42	0.01	73.86	0.01
312.5	80.06	0.02	81.45	0.02	90.84	0.03
2500	70.69	0.11	84.65	0.21	73.46	0.15

levels in mouse blood. The precision and accuracy were shown in Table 2. These results demonstrated that the precision and accuracy values were in the acceptance range (<15%).

3.2.3. Recovery, matrix effect and stability

The mean extraction recoveries determined using three replicates of QC samples at three concentration levels (the same concentrations as QC sample) in mouse blood were shown in Table 3. Methanol was used to extract maackiain, M-7-G, and M-7-S in blood samples. The result showed the recoveries were not less than 70% for these three analytes at low, medium, and high concentrations.

As for testing the matrix effects that may impact UPLC–MS analysis, the relative peak areas of these three analytes after spiking evaporated blood samples at three concentration levels were comparable to similarly prepared aqueous standard solutions. The results were reported in Table 4, which suggested that there was no measurable matrix effect observed.

The stabilities of maackiain and its metabolites in mouse blood were evaluated by analyzing three replicates of QC samples at three different concentrations following storage at 25 °C for 4 h, at 25 °C for 8 h, at −80 °C for 7 days, and after going through three freeze–thaw cycles (−80 °C and 25 °C). All the samples displayed 85–115% recoveries after various stability tests.

Table 4
Matrix effect related to the measurement of maackiain, M-7-G, and M-7-S at high, medium, and low concentrations.

C (nM)	ME (%)[a]			
	IS	Maackiain	M-7-S	M-7-G
39	136.0	92.9	98.1	103.5
312.5	94.7	87.5	84.8	82.3
2500	85.9	84.8	85.8	90.7

[a] Matrix effect expressed as the ratio of the mean peak area of an analyte spiked post-extraction to the mean peak area of the same analyte standards multiplied by 100.

3.3. Maackiain metabolites identification

M-7-G and M-7-S were identified as the major metabolites in pharmacokinetic study and Caco-2 cell culture mode transport experiment by comparing the retention times with those of standard metabolites obtained from biosynthesis, which were further confirmed by MS/MS spectral analysis. In Caco-2 cell culture experiment and pharmacokinetic study, two metabolites peaks were observed at 1.37and 1.67 min (Fig. 2). In MS analysis, the peak at 1.37 min showed pseudo molecular weight of *m*/*z* 459 $[M-H]^-$, which released a fragment of *m*/*z* 283 $[M-Glu-H]^-$ in MS^2 experiment (Fig. 3). Since there is only one hydroxyl group in the structure, the conjugate position for glucuronidation was assigned for position-7. Therefore the peak was identified as M-7-G. Similarly, the peak at 1.67 min was recognized as M-7-S.

3.4. Conversion factors (K) of extinction coefficients (EC)

We performed the glucuronidation and sulfation studies at three different concentrations (20, 10, and 5 μM) to calculate average *K* values. The results showed that the *K* value for M-7-G is 1.11 and for M-7-S is 0.84. These conversion factors were used to calculate the concentration of M-7-G and M-7-S using the standard curve of maackiain.

3.5. Application in mouse i.v. pharmacokinetics, Caco-2 experiment, and phase II metabolism studies

The validated method was employed to study the pharmacokinetic behaviors of maackiain in A/J mice. The mean plasma

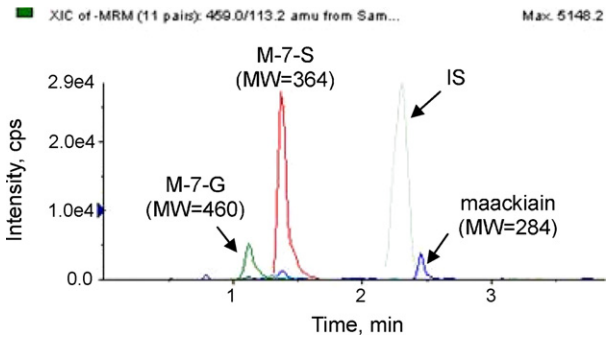

Fig. 2. The chromatograms of maackiain, M-7-G, M-7-S, and formononetin (IS) in a mouse blood sample. The retention time of M-7-S, M-7-G, IS, and maackiain were 1.37, 1.67, 2.32, and 2.68 min respectively. The molecular weight these four compounds were *m*/*z* 364, 460, 268, and 284.

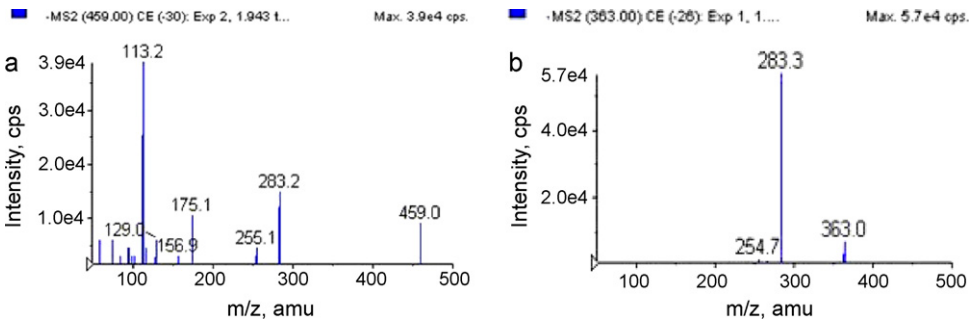

Fig. 3. The MS/MS chromatograms for M-7-G (a) and M-7-S (b). The results showed the molecular ion 459 $[M-H]^-$ and its MS^2 fragment at 283 $[M-H-Glu]^-$ for M-7-G and molecular ion 363 $[M-H]^-$ and its MS^2 fragment at *m/z* 283$[M-H-Sul]^-$ for M-7-S.

Table 5
Pharmacokinetics parameters of maackiain, M-7-S, and M-7-G after i.v. administration at 2.5 mg/kg dose ($n=5$).

Parameters	T_{max} (h)	C_{max} (μM)	AUC_{0-t} (h μM)	Half-life (h)	K (1/h)	V (l)	CL (l/h)
Maackiain	0.05 ± 0.11	10.73 ± 7.60	3.99 ± 1.98	7.37 ± 5.30	0.14 ± 0.09	544.27 ± 364.56	57.29 ± 31.48
M-7-S	0	0.92 ± 0.26	1.15 ± 0.54	–	–	–	–
M-7-G	0	7.46 ± 2.22	7.08 ± 1.87	–	–	–	–

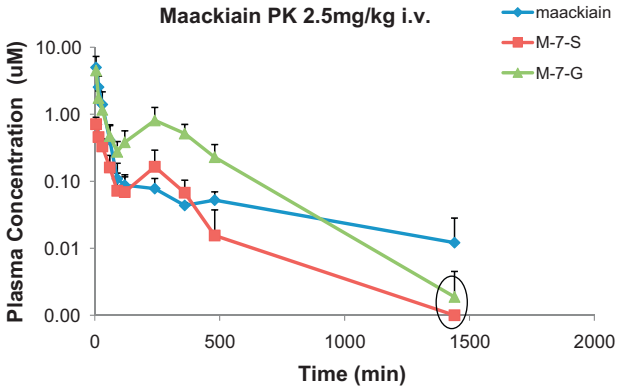

Fig. 4. Plasma concentrations of maackiain (diamond), M-7-G (square), and M-7-S (triangle) after i.v. administration of 2.5 mg/kg maackiain in A/J mice ($n=5$). Blood sample (20 μl) was spiked into KPI (20 μl), and IS (80 μl, formononetin in methanol, 1 μM). The mixture was vortexed and centrifuged for 15 min at 15,000 rpm. The supernatant was taken out and reconstituted in 80 μl of 50% methanol and centrifuged for another 15 min at the same speed. Then 10 μl of supernatant was injected into the UPLC–MS/MS system. Each point is average of five determinations and the error bars are standard deviations of the mean. The circled points represented data points where concentrations for some mouse blood samples fell below the LLOD.

concentration–time curves of maackiain and its metabolites (M-7-S, M-7-G) after i.v. administration (2.5 mg/kg dose) were shown in Fig. 4. For aglycone, the C_{max} was 10.73 μM, the T_{max} was

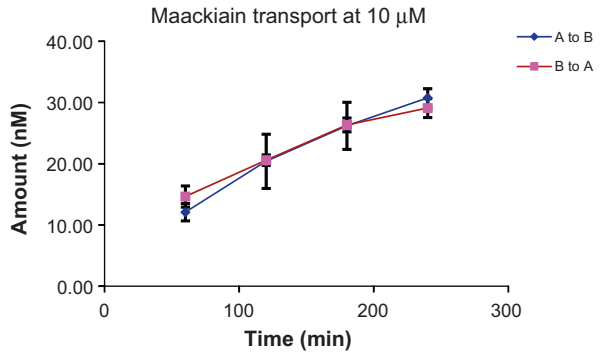

Fig. 5. Transport of maackiain in the Caco-2 cell culture model. The buffer used in both donor and receiver sides was HBSS (pH = 7.4). The donor side maackiain concentration was 10 μM for both apical to basolateral and basolateral to apical directions. The experiment was performed at 37 °C. Each data point is the average of three determinations. Bars are standard deviations of the mean.

0.05 h, and the AUC_{0-t} was 3.99 h μM (Table 5). M-7-S and M-7-G were detected as the major metabolites. The T_{max} of these two metabolites were 0 h, which suggested a rapid glucuronidation and sulfation of maackiain. The C_{max} of M-7-G and M-7-S were 7.46 and 0.92 μM respectively. The AUC_{0-t} values were7.08 h μM for M-7-G and 1.15 h μM for M-7-S, respectively. The observation suggested that glucuronide production level is higher than that of sulfate production after i.v. administration. Further studies are needed to

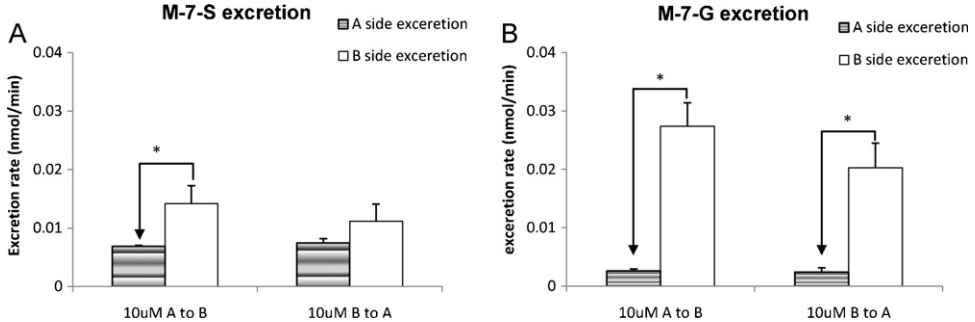

Fig. 6. Excretion rates of M-7-G and M-7-S in the Caco-2 cell culture model. The buffer used in both donor and receiver sides was HBSS (pH = 7.4). The donor side maackiain concentration was 10 μM for both apical to basolateral and basolateral to apical transport and metabolism studies. The experiment was performed at 37 °C. Each data point is the average of three determinations, and the error bar is the standard deviation of the mean. The asterisk (*) indicates a statistically significant difference between products and control ($p<0.05$, one-way ANOVA).

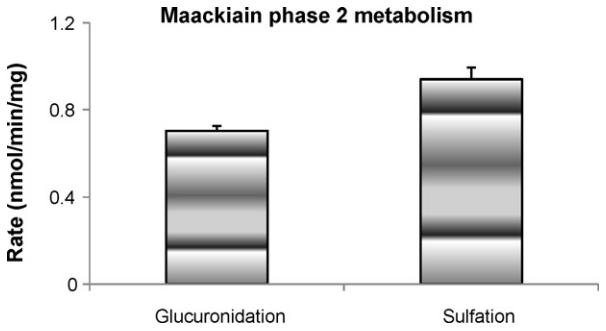

Fig. 7. Maackiain (1 μM) glucuronidation and sulfation rates in A/J mouse liver S9 fraction reaction. Each data point is the average of three determinations, and the error bar is the standard deviation of the mean.

determine its absolute bioavailability and explain the mechanisms responsible for its pharmacokinetic behaviors.

The validated method was also used to determine maackiain's transport and metabolites excretion in the Caco-2 cell culture model. The vectorial transport result showed that the apical to basolateral permeability (P_{ab}) of maackiain ($32.2 \pm 3.83 \times 10^{-6}$ cm/s) was similar to its basolateral to apical permeability (P_{ba}, $26.4 \pm 3.08 \times 10^{-6}$ cm/s). These high permeability values indicated that maackiain had good permeation across the Caco-2 cell monolayers at both directions (Fig. 5). The M-7-G and M-7-S were identified as the major metabolites. For excretion of M-7-S, when maackiain was loaded onto the apical side, the basolateral excretion rate was higher than that of apical excretion rate (Fig. 6A). When maackiain was loaded onto the basolateral side, the M-7-S excretion rates at both sides were identical. For M-7-G excretion, no matter how maackiain was loaded, the basolateral excretion was always more rapid (Fig. 6B). Further study is needed to explain these observations.

The validated method was also used to measure samples derived from *in vitro* metabolism studies of maackiain. The results revealed that maackiain (1.0 μM) glucuronidation and sulfation rate in A/J mouse liver S9 fraction were 0.70 ± 0.02 and 0.94 ± 0.05 nmol/min/mg protein, respectively (Fig. 7).

4. Conclusion

A rapid, sensitive and specific UPLC–MS/MS method had been developed and validated for the determination of maackiain, M-7-G, and M-7-S in plasma. The main advantages of this method are: (1) small blood volume (20 μl); (2) rapid analysis (4.0 min); (3) simple sample preparation procedure and good recovery; (4) minor matrix effect. Application of this analysis method to i.v. pharmacokinetic study showed that maackiain is extensively metabolized *in vivo* with glucuronides as the major metabolic pathway and sulfate as the secondary metabolic pathway. Since maackiain and maackiain metabolites can be analyzed simultaneously, each mouse can be used to derive a complete pharmacokinetic profile. This method is also valuable for human clinical study because it should allow higher sensitivity than reported here because large volume of blood sample is usually available in human, which may be used to concentrate the analytes before analysis.

Acknowledgement

The work was supported by NIH AT003203 to MH at University of Houston (Houston, TX) and to MY at Medical College of Wisconsin (Milwaukee, WI).

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