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Simple and Rapid Liquid Chromatography-Tandem Mass Spectrometry Analysis of Arctigenin and its Application to a Pharmacokinetic Study

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Abstract : Arctigenin is the main active ingredient of Fructus Arctii, which has been reported with a variety of therapeutic activities including anti-cancer, anti-inflammation, anti-virus, and anti-obesity effects. In this study, a simple and sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed and validated for the determination of arctigenin in rat plasma. The assay utilized a simple protein precipitation with methanol and the mobile phase consisted of 100% methanol and water containing 0.1% formic acid (65:35 v/v). Arctigenin and the internal standard (psoralen) were monitored using a positive electrospray turbo ionspray mode with multiple reaction monitoring transitions of *m/z* 373.2→136.9 and *m/z* 187.2→130.9, respectively, and total chromatographic run time was within 5 min. The lower limit of quantification (LLOQ) of arctigenin was 5 ng/mL in the rat plasma. The intra- and inter-day accuracy of arctigenin at LLOQ and matrix-matched quality control samples ranged 97.4 – 104.8% and 97.2 – 102.0%, respectively. The intra-day precision was within 4.80% and the inter-day precision was within 5.92%. Application of the present method was demonstrated through a pharmacokinetic study after intravenous and oral administration of arctigenin in male Sprague Dawley rats.

Keywords : Fructus Arctii, Arctigenin, LC-MS/MS, pharmacokinetics

Introduction

Arctigenin (AR) is a type of phenylpropanoid dibenzylbutyrolactone lignan, which is a main bioactive component of traditional Chinese medicine Fructus Arctii (fruit of *Arctium lappa L.*).[1-3] Recently, AR has been of particular research interest due to its various biologic activities. AR have demonstrated promising anti-cancer activities[4-8] and an early clinical trial investigated the safety of AR in pancreatic ductal adenocarcinoma patients.[9] In combination with quercetin, AR at low physiological doses provided a novel regimen with enhanced chemoprevention in prostate cancer.[10] AR also showed inhibitory effect of osteoclast differentiation,[11] ameliorating metabolic disorders,[12] decreased inflammatory reaction of acute inflammatory and inhibited B-cell and T-cell mediated allergic inflammation,[13-14] and inhibited replication of influenza A virus.[15] In *in vivo* and *in vitro* experiments neuroprotective activity of AR was reported.[16] AR exhibits cytotoxic and apoptotic effect in rheumatoid arthritis fibroblast-like synoviocytes through the activation of the mitochondrial pathway and suppression of NF-kB and Akt signaling pathways and may be promising as a new therapeutic agent for the treatment of rheumatoid arthritis.[17] Anti-obesity effect of AR has also been reported recently.[18]

Compared to the extensive reports on its pharmacologic activities, only limited information is available on the *in vivo* pharmacokinetic characteristics of AR. It has been suggested that AR may undergo extensive intestinal first-pass metabolism in *in vitro* and *in situ* models.[19] Methylated

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metabolites of AR, i.e., 3'-demethylarctigenin, 3'-demethyl-4'-dehydroxyarctigenin and 4', 4"-dihydroxylenterolactone were identified in the gastrointestinal tract.[2,20-22] Furthermore, following the oral administration of AR, rapid formation of the three major metabolites, arctigenin-4'-*O*-glucuronide, arctigenic acid, and 4-*O*-demethyl arctigenin were observed in the rat plasma.[19,23] Among these metabolites, arctigenic acid has been suggested to possess hypoglycemic activity.[24] However, most of the pharmacological activity is believed to be from AR and better understanding of the pharmacokinetic characteristics of AR is critical for further studies in order to be developed as a new therapeutic agent.

Several analytical methods such as high-performance liquid chromatography (HPLC) with UV,[1] fluorescence,[6] ESI/MS,[25], or MS/MS detection[26] have been reported for the determination of AR in *Arctium lappa L.* Nevertheless, limited methods are available for the analysis of AR for the *in vivo* pharmacokinetic studies including HPLC[27-28] and LC-MS/MS.[19,29] Moreover, most of these bioanalytical assays utilized laborious sample preparation procedures which included vacuum evaporation to dryness and reconstitution,[19,28-30] required extended run time,[28-29] or resulted in unsatisfactory sensitivity.[19,27-28] Therefore, in the present study we developed an LC-MS/MS method to determine AR in rat plasma by using simple protein precipitation with high sensitivity. The newly developed assay was fully validated and applied to a pharmacokinetic study of AR following intravenous and oral administration.

Experimental

Materials

AR and psoralen (internal standard, IS) were purchased from Enzo Life Sciences, Inc. (East Farmingdale, NY). Methanol, formic acid, and all other reagents were HPLC grades. Water used during the entire study was purified using a Milli-Q water purification system.

Animal study

All animal studies were conducted following the Guidelines for the Care and Use of Animals. The studies were approved by the Ethics Committee for the Treatment of Laboratory Animals at Wonkwang University.

Male Sprague-Dawley (SD) rats (8-9 weeks, 240-260 g; Hanilsirham dongmul center, Wan-ju, Korea) were kept in plastic cages with free access to standard diet and water. The animals were maintained at a temperature of 22-24°C with a 12 hr light-dark cycle and relative humidity of 50 ± 10%. The rats were anesthetized with intraperitoneal injection of 20 mg/kg Zoletil 50® (tiletamine HCl 125 mg/5 mL + zolazepam HCl 125 mg/5 mL) and cannulated with a polyethylene tubing (0.58 mm i.d., 0.96 mm o.d., Natsume, Tokyo, Japan) in the right jugular vein. After 24 hr of recovery, the animals were examined for its physical condition and the experiment was carried out only if the animal model was found to be stable. The rats were fasted 12 hr before AR dose.

For intravenous administration, AR (dissolved in normal saline containing 10% DMSO + 20% PEG 200 + 0.5% Tween 80, *w/w*) was given through the penile vein at 3 mg/kg (n=4). For oral administration, AR (suspended in 0.5% sodium carboxymethyl cellulose) was given to rats by oral gavage at 50 mg/kg (n=4). Venous blood samples were collected prior to and at 0.033, 0.0833, 0.167, 0.50, 1, 2, 3, 4, 6, and 8 hr of drug administration. Plasma samples were obtained by centrifugation of the blood samples at 10,000 × g for 10 minutes at 4°C and samples were stored at -20°C until analysis.

Calibration standards and quality control samples

The standard stock solutions of AR and IS were prepared in methanol at the concentration of 1.0 mg/mL. The IS working solution (50 ng/mL) was prepared by dilution of the stock solution with methanol. Plasma calibration samples were prepared by spiking 50 μL of the blank rat plasma with 50 μL of the IS working solution, 50 μL of the AR standard working solutions, and 50 μL of methanol to yield AR concentrations of 3000, 2000, 1000, 500, 100, 25, and 5 ng/mL. The quality control (QC) samples for plasma were prepared independently to provide high concentration QC (2500 ng/mL), medium concentration QC (1200 ng/mL), and low concentration QC (10 ng/mL). The calibration standards and QC samples were prepared and stored at -20°C until analysis.

Sample preparation

For plasma sample preparation, 100 μL of methanol and 50 μL of the IS working solution (50 ng/mL) were added to 50 μL of plasma samples as precipitation solvent. The mixture was mixed on a vortex mixer for 2 min followed by centrifugation for 10 min at 19,000 × g. The supernatant was then collected and 5 μL of the sample was injected onto the LC-MS/MS.

LC-MS/MS conditions

LC-MS/MS system consisted of API 4000 triple quadrupole mass spectrometer (AB MDS Sciex, Toronto, Canada) coupled with an Agilent 1100 HPLC system (Agilent, Santa Clara, CA, USA). Plasma samples were separated on a Kinetex C_{18} column (150 × 2.10 mm I.d., 2.6 μm) and the composition of mobile phase was a mixture of methanol and 0.1% of formic acid (65:35 v/v). The flow rate was set at 0.2 mL/min, and the column oven temperature was 40°C.

The electrospray ionization (ESI) source was operated in positive mode with the curtain and turbo-gas (all nitrogen) set at 30 and 6 psi, respectively. The turbo-gas temperature and the ion spray needle voltage were set at 400°C and 4500 V, respectively. The mass spectrometer was operated

in the multiple reaction monitoring (MRM) mode with a dwell time of 200 ms per MRM channel. The selected precursor/product ion pairs were m/z 373.2 → 136.9 for AR and m/z 187.2 → 130.9 for IS. The collision energy was set at 27 and 35 eV for AR and IS, respectively. Data acquisition was performed with Analyst 1.4 software (AB MSD Sciex, Toronto, Canada).

Assay validation

Linearity and sensitivity: The calibration curves were constructed by the weighted regression method (1/x) of peak area ratios of AR to internal standard vs. actual concentration. The determination of $r^2 > 0.999$ was desirable for the calibration curve. The lowest standard concentration on the calibration curve was accepted as the lower limit of quantification (LLOQ). The analyte peak of LLOQ sample should be identifiable, discrete, and reproducible with accuracy within ± 20% and precision ≤20%. The deviation of standards of other than LLOQ from the nominal concentration should be within ± 15%.

***Accuracy, precision, and recovery*:** The intra-day accuracy and precision of five replicates quality control (QC) samples were determined within one day at concentrations of 5, 10, 1200 and 2500 ng/mL for AR. The inter-day accuracy and precision of the QC samples were determined on five different days. The precision was expressed as the coefficient of variation at each concentration, and the accuracy was expressed as the percentage of the mean calculated vs. actual concentrations. The extraction recovery was determined for two different concentrations of AR at low concentration QC (10 ng/mL) and high concentration QC (2500 ng/mL) samples. AR was spiked in plasma and Millipore water separately, and that spiked in Milli-Q water served as un-extracted QC samples. Five replicates of each QC plasma sample were processed as usual and analyzed along with five replicates of un-extracted QC samples. Peak area ratios of the analyte after extraction of plasma samples were compared with those of un-extracted QC samples.

Stability: The stability of AR was examined under four different conditions using five replicates of low and high matrix matched QC samples. To assess the stability of AR in the rat plasma at room and storage temperature, low and high QC samples were left at 20°C for 4 hr and at -20°C for 2 weeks and AR concentrations were determined. The auto sampler storage stability was determined by storing the QC samples in the auto sampler at 4°C for 24 hr before being analyzed. The freeze-thaw stability was assessed by determining the remaining concentrations after low and high QC samples were subjected to three freeze–thaw cycles. The results were expressed as the percentage of the mean calculated over theoretical concentrations.

Results and Discussion

In the present study, an LC-MS/MS assay for the determination of AR concentration in rat plasma was developed. Simple protein precipitation and optimization of the chromatographic condition were able to improve assay sensitivity. Application of this assay was demonstrated through an *in vivo* pharmacokinetic study in rats. Although several analytical methods for determination of AR have been reported for an *in vivo* pharmacokinetic study, most of the methods require complicated sample preparation steps. Thus, a simple and sensitive LC-MS/MS assay for the determination of AR in biological matrix is necessary. The present method utilized a single step protein precipitation with a wide linear range and provided LLOQ of 5 ng/mL for rat plasma with accuracy within ± 20% and precision ≤ 20%.

Chromatography

Various mixture of organic solvents such as acetonitrile and methanol along with water, acetic acid, formic acid, and trifluoroacetic acid with altered flow rates (0.10 - 0.25 mL/min) were tested to optimize an effective chromatographic resolution for AR and IS. Finally, methanol was used as a protein precipitation solvent and the mobile phases comprising methanol: water with 0.1% formic acid (65:35, v/v) at a flow rate of 0.2 mL/min provided the best resolution of the peaks. The selected MRM transitions of protonated AR and IS and assay parameters are listed in Table 1 and the representative ion chromatograms are shown in Figure 1. Complete chromatographic run took 4.5 min.

One-step protein precipitation was used for the plasma sample preparation in the present method. Two organic solvents, i.e., methanol and acetonitrile were examined in the presence or absence of formic acid, acetic acid and triflouroacetic acid. Finally, methanol was found to be optimal, which can produce a clean chromatogram without interference from endogenous substances for AR and IS.

Linearity and sensitivity

The calibration curves were linear from 5 to 3000 ng/mL for AR in the rat plasma. The correlation coefficients (r^2)

Table 1. Observed MRM transitions and MS settings.

Compounds	MRM transition (m/z)	Retention time (min)	DP (V)	EP (V)	CE (V)	CXP (V)
Arctigenin	373.2 → 136.9	2.63	71	10	27	10
Psoralen (IS)	187.2 → 130.9	2.74	66	10	35	2

were consistently greater than 0.999 during the course of validation. The LLOQ for the assay was 5 ng/mL with accuracy of 102.0-104.8% and precision < 4.75% (Table 2). The present LLOQ is lower than those used for the *in vivo* pharmacokinetic studies of AR of 80 ng/mL,[27] 0.041 μg/mL,[28] and 25 ng/mL[19] in the rat plasma.

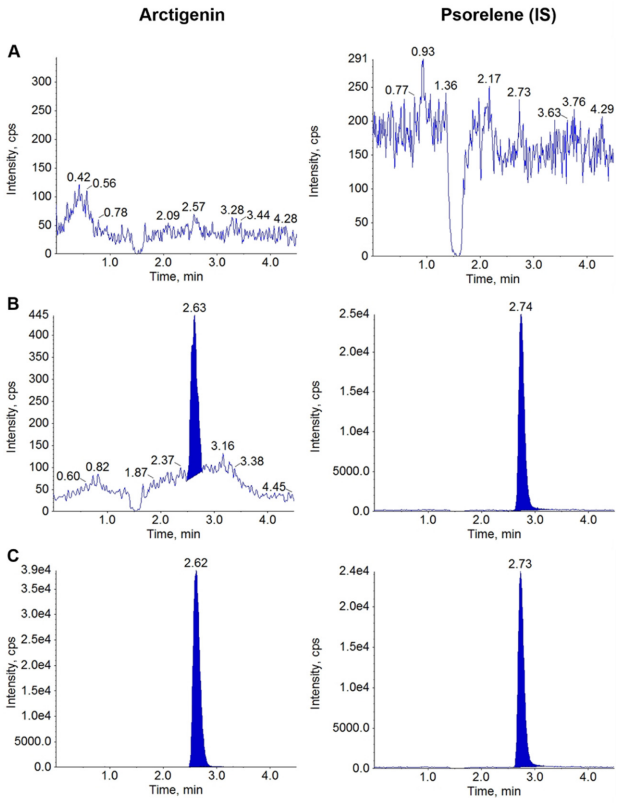

Figure 1. Representative chromatograms of arctigenin (left) and the internal standard (right) in the (A) blank plasma, (B) blank plasma spiked with arctigenin (5 ng/mL) and IS, and (C) plasma sample following intravenous injection of arctigenin.

Accuracy, precision, and recovery

Intra-and inter-day accuracy and precision data from five validation runs of QC samples at low, mid and high concentration levels and LLOQ are shown in Table 2. The intra- and inter-day accuracy ranged from 97.2% - 104.8%. The intra-day precision was 1.80% - 4.80% and the inter-day precision was 3.43% - 5.92%. These results indicated that the method had good accuracy and precision within the acceptance limit of ± 15% and < 15%, respectively. The percentage recovery of AR obtained from plasma at two concentration levels were 116.65 ± 9.29 and 101.96 ± 1.55% for the concentration 10 and 2500 ng/mL, respectively. These results indicated that the extraction efficiency for AR using protein precipitation was satisfactory, consistent and reliable.

Stability

The stability of AR in the rat plasma was investigated under various stability conditions to cover the possible exposure of samples during the analysis, storage and processing of samples. Table 3 shows that AR was stable in the auto sampler, freeze-thaw cycles, and short-term and long-term storage conditions.

Application to a pharmacokinetic study

The average plasma concentration-time profiles for AR after intravenous and oral administration are shown in Figure 2. Table 4 summarizes the average non-compartmental pharmacokinetic parameters of AR. The plasma concentration of AR rapidly declined following intravenous administration with terminal half-life ($t_{1/2}$) of 0.27 ± 0.02 hr. After oral administration, plasma concentration of AR increased rapidly and reached the maximum plasma concentration (C_{max}) of 69.03 ± 18.27 ng/mL within 5 min postdose. AR plasma concentrations then declined with the

Table 2. Intra- and Inter-day accuracy and precision of arctigenin assay in rat plasma.

Concentration (ng/mL)	Intra-day (n=5)			Inter-day (n=5)		
	Concentration found (ng/mL)	Accuracy (%)	Precision (%)	Concentration found (ng/mL)	Accuracy (%)	Precision (%)
5	5.32 ± 0.06	104.8	3.13	5.15 ± 0.16	102.0	4.75
10	10.4 ± 0.5	103.5	4.80	10.2 ± 0.3	100.6	3.96
1200	1160 ± 25	97.4	3.43	1154 ± 43	97.2	5.92
2500	2580 ± 57	102.6	1.80	2544 ± 87	101.8	3.43

Table 3. Stability of arctigenin in rat plasma (n=5).

Concentration (ng/mL)	Percentage over theoretical concentration (%)			
	Auto sampler stability (24 hr, 4°C)	Freeze-thaw stability (3 cycles, -20°C)	Short-term stability (4 hr, 20°C)	Long-term stability (2 wk, -20°C)
10	95.0 ± 2.5	103.2 ± 1.5	100.8 ± 4.8	101.7 ± 3.1
2500	95.3 ± 2.8	99.1 ± 1.6	93.5 ± 1.9	95.9 ± 2.0

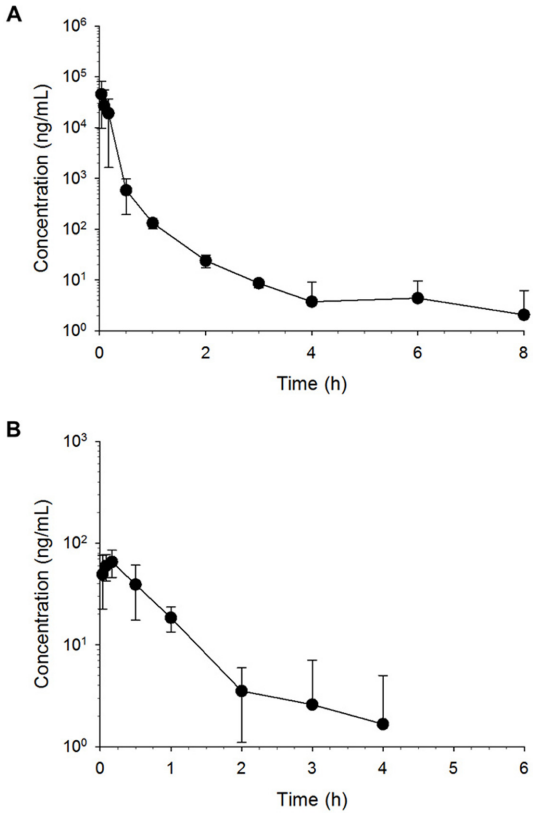

Figure 2. Average plasma concentration vs. time profiles of arctigenin after (A) intravenous (IV) administration at dose of 3 mg/kg (n = 4) and (B) oral (PO) administration at dose of 50 mg/kg (n = 4) in rats. Data represent the mean ± SD.

Table 4. Non-compartmental pharmacokinetic parameters of arctigenin following intravenous (IV, 3 mg/kg) and oral (PO, 50 mg/kg) administration in rats.

Pharmacokinetic parameters	Route of administration	
	IV (n = 4)	PO (n = 4)
$t_{1/2}$ (hr)	0.27 ± 0.02	0.50 ± 0.06
C_0 or C_{max} (ng/mL)	53175 ± 29278	69.03 ± 18.27
AUC_{last} (ng·hr/mL)	9198 ± 4720	42.40 ± 18.45
AUC_{inf} (ng·hr/mL)	9204 ± 4717	53.09 ± 19.01
V_z or V_z/F (mL)	156.1 ± 70.0	1023429 ± 401849
CL or CL/F (mL/hr)	405.9 ± 198.2	1418023 ± 533344
V_{ss} (mL)	52.3 ± 24.4	-
F (%)	-	0.035%

$t_{1/2}$ of 0.50 ± 0.06 hr. The area under the plasma concentration vs. time curve (AUC_{inf}) was 9204 ± 4717 ng·hr/mL for the intravenous administration and 53.09 ± 19.01 ng·hr/mL for oral administration. The oral bioavailability of AR was estimated to be 0.035%.

The low oral bioavailability of AR found in the present study was consistent with a previous literature report.[31] It has been reported that AR plasma concentrations were mostly lower than the quantification limit after oral administration at doses lower than 12 mg/kg, and the pharmacokinetic parameters could not be estimated.[31] The low bioavailability may be associated with extensive intestinal as well as hepatic first-pass metabolism suggested from *in vitro* and *in situ* studies.[19,31] The major metabolites were identified as arctigenic acid and arctigenin-4'-*O*-glucuronide after both intravenous and oral administrations of AR in rats.[23,31] Although arctigenic acid also showed hypoglycemic activity in a recent study,[24] the contribution of the metabolites to the various *in vivo* pharmacological effects of AR needs to be evaluated by further studies.

Conclusions

A simple, rapid, and sensitive LC-MS/MS assay for quantification of arctigenin in rat plasma was developed. The assay utilized one-step protein precipitation and achieved superior sensitivity for *in vivo* pharmacokinetic studies of AR (LLOQ = 5 ng/mL) with short chromatographic run time (4.5 min). The developed assay was fully validated using matrix matched QC sample and provided a linear dynamic range 5-3000 ng/mL for rat plasma. This assay was successfully applied to an *in vivo* study to determine pharmacokinetic characteristics of arctigenin in rats following intravenous and oral administration. The LC-MS/MS assay and pharmacokinetics of AR in rats in the present study may provide useful tool for further preclinical studies as well as clinical studies of AR.

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