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Development and validation of an UPLC-MS/MS method for the quantification of columbin in biological matrices: Applications to absorption, metabolism, and pharmacokinetic studies

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Abstract

The aim of this study is to develop a sensitive UPLC-MS/MS method to quantify columbin in biological sample. Chromatographic separation was accomplished using Waters UPLC BEH C₁₈ column with acetonitrile and 0.1% of formic acid in water as the mobile phases. The mass analysis was performed on an API 5500 Qtrap mass spectrometer *via* multiple reaction monitoring (MRM) with positive scan mood. The one-step protein precipitation by methanol was used to extract the analyte from blood samples. The results showed that the linear response range for columbin was 1.22–2,500 nM. The intra and inter day variances were less than 15% and the accuracy was in acceptable range (85–115%). The analysis was done within 3.0 min, and only 50 µL of blood was needed. The validated method was used to determine the pharmacokinetic profile of columbin in Wistar rats, and its transport characteristics in the Caco-2 cell culture model. The results showed that columbin was poorly bioavailable (2.8% p.o. and 14% i.p.) in rats, but its transport was rapid across the Caco-2 cell monolayers, suggesting that extensive first-pass metabolism in the liver was the likely reason for its poor bioavailability. The results revealed that the validated method can be used for columbin analysis in both bioequivalent buffer and blood.

Keywords

Columbin; Pharmacokinetic; Caco-2 transport; UPLC-MS/MS

1. Introduction

Columbin, (Fig. 1), a diterpenoid furanolactone, is found in many plants, but it was first isolated from *Tinospora bakis* [1]. Pharmacological studies showed that this diterpenoid has multiple attractive biological activities, including anti-carcinogenesis [2,3], anti-inflammatory [2,3], and anti-hyperlipidemia (by reducing cholesterol uptake) [4]. *In vivo* chemoprevention studies indicated that columbin was actively against human colon cancer carcinogenesis [5,6]. In addition to be bioactive, columbin may also impact the metabolism

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of co-administered drugs, since it was shown to affect the sleeping time of anesthetized mice [7]. As an active chemical component in Chinese herb *Tinospora sagittata* (Oliv.) Gagnap. or *Tinospora capillipes*, columbin is also commonly used as a marker compound for the purpose of quality control [2,3].

In order to enhance the development potentials of columbin as a chemopreventive agent, there is a need to further understand the *in vitro* and *in vivo* absorption and clearance characteristics of columbin. There are several papers on columbin pharmacokinetics studies using various detection methods [2,3]. But these methods are not sensitive. In addition, the information on Caco-2 transport characteristics as well as the metabolism (e.g., phase I, glucuronidation) was not found in the published literatures. Therefore, the purpose of this study is to (1) establish a sensitive and reliable UPLC-MS/MS method to determine columbin concentrations in different matrixes; (2) investigate its pharmacokinetic behaviors in rats; and (3) determine the transepithelial transport characteristics of columbin using the Caco-2 cell culture model.

2. Experimental

2.1. Chemicals and reagents

Columbin were purchased from Chinese National Institutes for Food and Drug Control. Cloned Caco-2 cells (TC7) were a kind gift from Dr. Monique Rousset of INSERM U178 (Villejuif, France). Oral suspending vehicle was purchased from Oral-plus (Paddock Laboratories, USA). Saccharolactone, alamethicin, magnesium chloride, phosphoric acid, Hanks' balanced salt solution, sodium citrate, potassium phosphate dibasic, formononetin, were purchased from Sigma (St. Louis, MO). All other materials (typically analytical grade or better) were used as received.

2.2. Instruments and conditions

An API 3200 Qtrap triple quadrupole mass spectrometer (Applied Bio system/MDS SCIEX, Foster City, CA, USA) was used to determine the concentrations of columbin in HBSS buffers and blood by MRM (multiple reaction monitoring) scan type with positive ionization mode.

UPLC conditions were: system, Waters Acquity TM with diode array detector (DAD); column, Acquity UPLC BEH C₁₈ column (50 × 2.1 mm L.D., 1.7 μm, Waters, Milford, MA, USA); mobile phase A, 0.1% formic acid; mobile phase B, 100% acetonitrile; gradient, 0–0.5 min, 0–5% B, 0.5–1.0 min, 5–50% B, 1.0–1.5 min, 50–95% B, 1.5–3.0 min, 95% B; flow rate, 0.55 mL/min; column temperature, 45 °C; and injection volume, 10 μL. Mass spectrometer and UPLC conditions for analyzing formononetin was same as the above method.

2.3. Method validation

2.3.1. Calibration curve and QC samples—Stock solution of columbin was prepared in DMSO at a concentration of 20 mM. The stock solution of formononetin (I.S.) was prepared in acetonitrile at a concentration of 10 mM. Calibration standards were prepared in

50 % acetonitrile by diluting a stock solution of columbin to final concentrations of 2500.00, 1250.00, 625.00, 312.50, 156.00, 78.00, 39.00, 19.50, 9.75, 4.88, 2.44, 1.22, and 0.61 nM respectively. To prepare standard curve in buffer, the stock solution was diluted into buffer directly (200 μ L) and 20 μ L of I.S. was added. To prepare standard curve in blood, blank blood (50 μ L) was mixed with 50 μ L of standard curve samples prepared in 50% acetonitrile and 160 μ L of I.S. in acetonitrile (0.2 μ M). After centrifugation at $20,000 \times g$ for 15 min, the supernatant was transferred to a new tube and the solvent was removed under a flow of nitrogen and the residue was reconstituted in 80 μ L of 50% acetonitrile and centrifuged at $20,000 \times g$ for 15 min for injection. The quality control (QC) samples were prepared at low (9.77 nM), medium (156.25 nM), and high (1250 nM) concentrations in the same way as the blood samples for the calibration standards.

2.3.2. Linearity—The linearity of each calibration curve was determined by plotting the ratio of the peak areas of columbin to internal. A least-squares linear regression method ($1/x^2$ weight) was used to determine the slope, intercept and correlation coefficient of linear regression equation.

2.3.3. Specificity and LLOD—(The lower limit of detection) The specificity of the method was measured by analysis of different blood samples of different origin for interference at the retention times of the columbin. Specificity was assessed by comparing the peak of a analyte in blank blood sample to that in a blank blood sample spiked with analyte at 2.44 nM. The LLOD was defined based on a signal-to-noise ratio of 10:1.

2.3.4. Precision and accuracy—The intra/inter-day precision and accuracy of the method were determined by measuring three different concentrations of quality control (QC) samples on the same day or in three different days.

2.3.5. Extraction recovery and matrix effect—The extraction recovery of columbin was determined by comparing the peak areas obtained from blank blood spiked with the analyte before the extraction with those from samples to which analyte was added after the extraction. Matrix effect was determined by comparing the peak areas of blank blood extracts spikes with the analyte with those of the standard solutions dried and reconstituted with mobile phase A.

2.3.6. Stability—Short-term (25 °C for 4 h), long-term (−20 °C for 14 days), and freeze-thaw cycle (three cycles) stabilities were determined.

2.4. Transport study in the Caco-2 cell culture model

2.4.1. Cell culture—Caco-2 transport study has been routinely done by our lab for almost two decades. The culture conditions for growing Caco-2 cells had been described previously [8,9]. To seed the cells, we used 3 μ m porous polycarbonate cell culture inserts from Nunc (distributed by VWR International), which has a surface area of 4.2 cm². The seeding density (60,000 cells/cm²), growth media (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum), and quality control criteria were all

implemented according to previous published reports [10]. Caco-2 TC7 cells were fed every other day, and cell monolayers were ready for experiments from 19 to 22 days post-seeding.

2.4.2. Transport experiments—To determine the permeability of columbin, the Caco-2 cell monolayers were washed three times with HBSS. The transepithelial electrical resistance (TEER) values were measured and only those wells with TEER values higher than $420 \Omega/\text{cm}^2$ were used. The monolayers were incubated with the HBSS buffer for 1 h before the experiment. Then, 2.5 mL of columbin solution (10 μM) in HBSS or blank HBSS was loaded onto the apical or basolateral side respectively. Five samples (0.5 mL) were taken at different times (0, 60, 120, 180, 240 min) from both sides and same volume of columbin solution or blank HBSS was replaced after each sampling. 50 μL of internal standard solution (0.2 μM formononetin) was immediately added to 200 μL of samples. The mixture was centrifuged at $20,000 \times g$ for 15 min, and the supernatant was analyzed by UPLC-MS/MS.

2.4.3. Data analysis in the Caco-2 cell culture model—The apparent permeability coefficient (P) was determined by the following relationship

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

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

2.5. Metabolism of columbin in phase I and II reaction systems

2.5.1. Phase I reaction system—The incubation procedures for Phase I reaction using liver microsomes were the same as those published previous by our laboratory [11]. Briefly, columbin (10 μM) was mixed with the rat liver microsomes (Wistar male rat, final concentration $\approx 0.36 \text{ mg protein/mL}$), NADP (2.61 mM), 6-DP (6.6 mM), MgCl_2 (6.6 mM), and 6-DPD in 5 mM sodium citrate solution, in a 50 mM potassium phosphate buffer (pH 7.4, total volume 200 μL). The mixture was incubated at 37 $^\circ\text{C}$ for 1 h and the reaction was terminated by adding 50 μL formononetin (0.2 μM) in acetonitrile. The samples were vortexed for 30 s, centrifuged at $20,000 \times g$ for 10 min, and 10 μL of supernatant was injected into UPLC-MS/MS system for metabolite screening. EMS (enhanced mass spectrometer) mode was used to search for possible phase I metabolite of columbin. Positive control experiment was done by determining the concentrations of 6 β -hydroxytestosterone and testosterone after 10 μM testosterone was incubated in the same conditions.

2.5.2. Glucuronidation reaction system—The incubation procedures for glucuronidation reaction using liver microsomes were the same as those published previous by our laboratory [12]. Briefly, columbin (10 μM) was mixed with the rat liver microsomes (Wistar male rat, final concentration $\approx 0.36 \text{ mg protein/mL}$), magnesium chloride (0.88 mM), saccharolactone (4.4 mM), alamethicin (0.022 mg/mL), and UDPGA (3.5 mM, add

last) in a 50 mM potassium phosphate buffer (pH 7.4, total volume 200 μ L). The reaction system was incubate at 37 $^{\circ}$ C for 1 h. To terminate the reaction, 50 μ L of formononetin (0.2 μ M, as the internal standard) solution in acetonitrile was added. The samples were then vortexed for 30 s, and centrifuged at $20,000 \times g$ for 10 min, and 10 μ L of supernatant was injected into UPLC-MS/MS system for analysis. EMS mode was used to search for possible glucuronide of columbin. Positive control experiment was done by determining the concentrations of genistein and genistein 7-*O*-glucuronide after 10 μ M genistein was incubated under the same conditions.

2.6. Pharmacokinetic study

2.6.1. Animals—Male Wistar rats (250–270 g, 8–10 weeks old) were from Harlan Laboratory (Indianapolis, IN) and kept in an environmentally controlled room (temperature: 25 ± 2 $^{\circ}$ C, humidity: $50 \pm 5\%$, 12 h dark–light cycle) for at least 1 week before the experiments. The rats were fasted overnight with free access of water before the day of the experiment. The animal protocols used in this study were approved by the University of Houston's Institutional Animal Care and Uses Committee.

2.6.2. Experimental design—Three groups of rats were treated as following: i.v. injection of columbin in EtOH and PEG-300 (1:1) was administrated through tail vein at dose of 20 mg/kg. Intraperitoneal (i.p.) injection of columbin in EtOH and PEG-300 (1:1) was administrated at dose of 20 mg/kg. An oral gavage of columbin suspended in oral suspension vehicle was given to rats at dose of 50 mg/kg. Blood samples (50–100 μ L) were collected by snipping the tail into heparinized tubes at 0, 5, 15, 30, 45, 60, 120, 240, 360, 480 and 1440 min for i.v. administration, or at 0, 15, 30, 60, 120, 180, 240, 360, 480, 1440 min for oral dosing or i.p. injection. The blood samples were stored at -20 $^{\circ}$ C until analysis.

2.6.3. Blood sample preparation—The blood samples were prepared according to the method described in section 2.3.1. Briefly, 50 μ L of 50% of acetonitrile was spiked into 50 μ L of blood sample, which was extracted by 160 μ L of I.S. in acetonitrile. The mixture was vortexed for 1 min and the supernatant after centrifugation at $20,000 \times g$ for 15 min was transferred into a new tube. The solvent was removed with a stream of nitrogen and the residue was reconstituted in 80 μ L of 50% acetonitrile, which was again centrifuged ($20,000 \times g$ for 15 min) for LC–MS injection.

2.6.4. Data analysis—WinNonlin 3.3 was used for pharmacokinetic analysis. The non-compartmental approach was applied for analysis of the data following i.v., i.p., and oral administration of columbin.

3. Results and discussion

3.1. Method development

Different mobile and columns were tested to enhance the peak shape of columbin. Methanol, acetonitrile, 2.5 mM ammonia acetate (pH 7.6), 0.1–0.5% formic acid, and 100% water were tested as potential mobile phases. Both C₁₈ and C₈ columns were evaluated as stationary phases. Based on the intensity of the signal, and the shape of the peak, acetonitrile and 0.1%

formic acid and C₁₈ column were found to be the optimal mobile and stationary phases, respectively. The column temperature was 45 °C and the flow rate at 0.55 mL/min. A representative chromatogram is in Fig. 2A and the MS/MS spectra is in Fig. 2D.

For MS analysis, to improve the sensitivity of columbin, both positive and negative scan modes were evaluated. Based on the intensity of the analytes, positive scan mode was found to be more sensitive. The compound and instrument dependent parameters were optimized by tuning with infusion of a columbin solution (1.0 µM). The main working parameters for mass spectrum were set as follows: ionspray voltage, 5.5 kV; ion source temperature, 400 °C; gas1, 30 psi; gas2, 40 psi; curtain gas, 10 psi. Formononetin was used as an internal standard for the analysis of columbin in blood and cell culture medium samples. The transitions of m/z 359 → m/z 175 for columbin, m/z 269 → m/z 197 for formononetin (IS). The compound dependent parameters in MRM mode for columbin and other compounds were summarized in Table 1.

3.2. Method validation

3.2.1. Specificity—To increase the specificity, MRM (multiple reaction monitoring) scan type was used in this method. The results showed that there is no significant interference in the chromatogram. (Fig. 2).

3.2.2. Linearity and LLOD (lower limit of detection)—The standard curve was linear from 1.22 nM to 2500.00 nM ($R^2 > 0.99$) in blood. The accuracy of each standard sample was in the acceptable range (85.7–103.7%). The LLOD (lower limit of detection) was 0.6 nM in blood.

3.2.3. Accuracy and precision—Intra/inter-day precisions, and accuracy were determined using QC samples at three concentrations spiked in the blank rat blood. The results, which are shown in Table 2, demonstrated that the precision and accuracy values were within the acceptance range (<15%).

3.2.4. Recovery, matrix effect and stability—The mean extraction recoveries determined using three replicates of QC samples at three concentration levels in rat blood were found $84.1 \pm 6.2\%$, $76.3 \pm 3.1\%$, and $79.4 \pm 2.9\%$ at 9.77, 156.25 and 1250 nM, respectively. For matrix effect, the peak areas of columbin after spiking evaporated blood samples at three concentrations were comparable to similarly prepared aqueous standard solutions (ranged from 94.3% to 106.7%), suggesting that there was no measurable matrix effect that interfered with columbin determination in the rat blood.

Stability was measured by spiked columbin into blank blood at three different final concentrations at 9.77, 156.25 and 2500 nM, which were storage at 25 °C for 4 h, –20 °C for 14 and within three freeze–thaw cycles. All the samples displayed 90–110% recoveries after various stability tests. These results indicated that columbin was stable at these conditions.

Taken together, the above results showed that a sensitive, reproducible, and robust method for the analysis of columbin in multiple sample matrixes has been developed, and validated for use in the analysis of columbin in rat blood and Caco-2 transport media.

3.3. Method comparison

An LC–MS method to quantify columbin in the plasma has been published previously [13]. However, there are multiple improvements in this method when compared with the published one. Firstly, the limit of quantification in this method is 1.2 nM, while it was 5 ng/mL (13.9 nM) in the published method. Secondly, only 50 μ L of blood is needed in this method, while 100 μ L of blood was used in the old method. Thirdly, the extraction solvent used in here is acetonitrile, which is used as mobile phase. In the previously published method, methyl *tert*-butyl ether (MTBE) was used as the extraction solvent. There are two disadvantages using this MTBE as extraction solvent: (1) it is difficult to control the accuracy because MTBE evaporates rapidly; (2) interference may added to the sample as MTBE was not used as mobile phase. Therefore, the current method is better than the published one.

3.4. Transport study in cell culture models

This validated method was used to quantify columbin in the transport study in the Caco-2 cell culture model. The results showed that the absorptive permeability (P_{ab} , transport from apical to basolateral side) of columbin is 2.60×10^{-5} cm/s), which is a relative high number that may predict to more than 70% of absorption *in vivo* according to the reference [14,15]. The efflux permeability (P_{ba} , transport from basolateral to apical) of columbin was also found to be high (2.31×10^{-5} cm/s, Fig. 3). The efflux ratio (P_{ba}/P_{ab}), which indicates the efflux of the tested drug, of columbin is close to 1-fold (0.88). These results indicated that passive diffusion is the likely mechanism for transport of columbin across the Caco-2 cell monolayers.

3.5. Metabolism studies

The validated method was used to quantify columbin in the metabolism studies. The results of columbin metabolism in rat liver microsomes studies showed that columbin could not be metabolized by CYP450 and UGT enzymes, even though two positive control experiments showed that 10 μ M testosterone (CYP control) and genistein (UGT control) had been thoroughly metabolized in the appropriate reaction systems. These results suggested that columbin could not be extensively metabolized by CYP and UGT enzymes, which are two of the most common metabolism pathways.

3.6. Pharmacokinetic studies

This validated method was used in the pharmacokinetic studies in rats. The mean blood concentration-time curves of columbin after i.v., i.p., and p.o. administration are presented in Fig. 4. The pharmacokinetic parameters calculated by the non-compartmental method. The fitted pharmacokinetic parameters were shown in Table 3. The results indicated that columbin was reasonably rapidly absorbed following oral and i.p. administration with T_{max} of 20 min for i.p. and 60 min for oral administration. However, the drug had poor bioavailability (F%) after either the oral or i.p. administration. Based on a F% of 14% for i.p. and 2.8% for p.o. administration, we could conclude that columbin was extensively metabolized in the liver, with additional contribution from the intestine, as F% after p.o. was even smaller than the F% after i.p. administration. Since we couldn't find any metabolite in

the CYP and UGT metabolism studies with rat liver microsomes, further study is needed to investigate the metabolism pathway of columbin in rat.

4. Conclusion

A rapid, sensitive, and specific UPLC-MS/MS method had been developed and successfully applied for analyzing columbin in aqueous media, HBSS Caco-2 cell study media, microsomal media, and blood samples. The precision, extraction recovery, matrix effect and stability of this method had been determined and the method was validated. Using this newly developed assay, we were able to determine that columbin is poorly bioavailable following oral or i.p. administration. More studies are needed to identify the mechanism of poor oral and i.p. bioavailabilities of columbin.

Abbreviations

UPLC	ultra-performance liquid chromatography
IS	internal standard
DP	declustering potential
CE	collision energy
CXP	collision cell exit potential
AUC	area under the curve
QC	quality control
LLOQ	lower limit of quantification
MPA	mobile phase A
MPB	mobile phase B

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Fig. 1.
Chemical structures of columbin.

Fig. 2. Representative MRM chromatograms of columbin and I.S. in rat plasma (A). B and C are blank blood (B) or blank blood spiked with columbin (C) (2.44 nM) respectively. D is the MS/MS spectra of columbin.

Fig. 3.

Bidirectional transport of columbin across Caco-2 cell monolayers. The buffer used in both donor and receiver sides was HBSS (pH 7.4). The donor side concentrations of columbin (both apical and basal sides were tested) were always 10 μ M. Experiments were performed at 37 °C. Each data point represents the average of three replications. Error bars indicate the standard deviation.

Fig. 4. Blood concentrations of columbin after i.v., i.p, and p.o. administration in SD rats ($n = 6$).

Table 1

Compound dependent parameters for columbin and formononetin in MRM mode for UPLC-MS/MS analysis.

Analyte	Q ₁ mass(U)	Q ₃ mass(U)	Dwell time (ms)	DP(V)	CEP(V)	CE(V)	CXP(V)
Columbin	359	175	100	40	13	40	3
Formononetin	269	197	100	67	17	49	3

Table 2

Intra-day and inter-day precision of columbin.

Linear range (nM)	UPLC-MS/MS					
	First day (<i>n</i> = 5)		Second day (<i>n</i> = 5)		Third day (<i>n</i> = 5)	
Con(nM)	Accuracy (Bias, %)	Precision (CV, %)	Accuracy (Bias, %)	Precision (CV, %)	Accuracy (Bias, %)	Precision (CV, %)
2,500.00-1.22	1250.00	87.5 4.3	90.49 2.30	86.87	5.17	
156.25	98.3 8.9	96.37 8.59	96.60	8.87		
9.77	103.7 4.2	102.28 6.37	96.43	6.56		

Table 3

Pharmacokinetic parameters of columbin in rat plasma ($n = 4$) after i.v.(20 mg/kg), i.p.(20 mg/kg) and p.o. administrations (50 mg/kg).

Pharmacokinetic parameter	i.v. (mean $\pm$ SD)	p.o. (mean $\pm$ SD)	i.p. (mean $\pm$ SD)
$T_{\max}$ (min)	7.50 $\pm$ 2.66	60.00 $\pm$ 0.00	20 $\pm$ 8.66
$C_{\max}$ (μ M)	3.65 $\pm$ 1.99	0.0395 $\pm$ 0.0142	0.0476 $\pm$ 0.0157
$T_{1/2}$ (min)	329.60 $\pm$ 88.90	605.59 $\pm$ 24.52	444.58 $\pm$ 164.69
Dose adjusted AUC _{last}	659.06 $\pm$ 90.90	16.75 $\pm$ 0.43	86.35 $\pm$ 14.58
Dose adjusted AUC _{inf} (min μ M)	715.35 $\pm$ 98.97	19.73 $\pm$ 1.21	93.26 $\pm$ 10.23
K_e	0.0037 $\pm$ 0.0030	0.0012 $\pm$ 0.0000	0.0018 $\pm$ 0.0008
CL	0.0474 $\pm$ 0.0179	984.26 $\pm$ 26.91	20610.98 $\pm$ 2977.07
V	12.66 $\pm$ 6.88	854531.58 $\pm$ 57167.2	7675466.96 $\pm$ 87256.78
Bioavailability (%)	–	2.76 $\pm$ 0.43	14.15 $\pm$ 11.25