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The pharmacokinetic, bioavailability and Excretion of columbianetin acetate and its metabolite columbianetin in rat plasma by LC-MS/MS after administration of columbianetin acetate and *Angelicae pubescentis* Radix extract

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Abstract

Angelicae pubescentis Radix (APR) has been widely used in clinic for the treatment of rheumatoid arthritis. Columbianetin acetate and its metabolite columbianetin have various biological activities. A sensitive, accurate and precise HPLC–MS/MS method was established for simultaneous determination of columbianetin acetate and columbianetin in rat plasma. It was found that columbianetin acetate was rapidly and widely distributed in rats, and eliminated rapidly from plasma. Columbianetin acetate could be metabolized into columbianetin *in vivo*. Absolute bioavailability of pure columbianetin acetate is 7.0 ± 4.3%. Other co-existing ingredients in APR extract could increase the concentration of its metabolite columbianetin in plasma and this was caused by columbianetin-β-D-glucopyranoside. Cumulative excretion of columbianetin acetate in urine accounted for 0.0109 ± 0.0067% of total dosage. The cumulative amounts of columbianetin acetate in the feces present 9.32 ± 6.63% of the total dose. Columbianetin acetate was mainly excreted in the feces.

Keywords: Angelicae pubescentis Radix; Columbianetin acetate; columbianetin; LC–MS/MS; pharmacokinetics; Excretion

1. Introduction

Traditional Chinese medicines (TCMs) have been used clinically for preventing and treating diseases for thousands of years. By virtue of its clinical application over such a long period of time, a large amount of TCMs have been proven to be effective. Unlike chemical medicine, TCM is a complex system with various active ingredients in which one active ingredient would interact with the others. According to the results of several previous researches, there are significant differences between pharmacokinetic properties of the active components in their pure forms and those in herbal medicines [1, 2] or even in formulas [3].

Angelicae pubescentis Radix (APR), the root of *Angelica pubescens* (Apiaceae) Maxim. f. *biserrata* Shan et Yuan is officially listed in the Chinese Pharmacopoeia (National Commission of Chinese Pharmacopoeia, 2010). APR has been widely used in clinic for centuries as a known TCM for the treatment of rheumatoid arthritis both in Chinese herbal medicine [4, 5] and Chinese patent medicine [4]. Researches have shown that APR extracts exhibit anti-inflammatory and analgesic effects [6]. The coumarins from APR could prolong the hypnotic duration of pentobarbital sodium [7]. Additionally, there are reports that APR has been used clinically in the treatment of influenza A [8], migraine [9] osteoporosis, [10] and stroke [11].Coumarins are the major bioactive principles in APR [12, 13]. Columbianetin acetate (Fig. 1) is one of the major coumarins in APR[14], and columbianetin (Fig. 1) is a major bioactive coumarin. These two coumarins were both firstly isolated from APR in 1987 [12].

According to reports in literature, columbianetin acetate has been shown to have

multiple biological activities including anti-tumor [15], anti-platelet formation [13], anti-inflammatory and analgesic [16]. Columbianetin has also been found to inhibit platelet aggregation and lipid peroxidation [17], inhibit histamine release [18, 19], inhibit activation of the virus-genome and the viabilities of Raji cells [15] and anti-inflammatory activity [19] and anti-pathogen effects [20]. It was usually reported that some compounds may be metabolized to analogues *in vivo* after administration [21]. Columbianetin acetate and columbianetin are analogues with the same mother nucleus. It was necessary to investigate the relation of columbianetin acetate and columbianetin *in vivo.* At present, pharmacokinetic studies on columbianetin acetate after oral administration of APR extract in rats have been reported in our previous reseach [22]. However, there is no report on the study of *in vivo* pharmacokinetics，excretion and bioavailability of pure columbianetin acetate. To our knowledge, the influence of other co-existing ingredients in APR extract on columbianetin acetate and columbianetin was also not reported.

In present study, a simple and sensitive high performance liquid chromatography–tandem mass spectroscopy (LC–MS/MS) has been developed for the quantitative determination of columbianetin acetate and its metabolite columbianetin in rat plasma. The newly established LC–MS/MS method was successfully applied to the pharmacokinetics, oral bioavailability and excretion study after administration of columbianetin acetate and APR extract to rats.

2. Experimental

2.1. Chemicals and reagents

Methanol of HPLC grade, ethyl acetate and petroleum ether of analytical grade were purchased from Tianjin Concord Science Co. Ltd., (Tianjin, China). Acetonitrile of Chromatographic grade for liquid chromatography was purchased from Merck (Darmstadt, Germany). Ammonium acetate (purity>99.99%) was purchased from Tianjin Guangfu Institute of Fine Chemicals (Tianjin, China). Warfarin (purity > 98%) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). APR was obtained from Anguo city (Hebei, China). The species were authenticated by Professor Lin Ma.Tianjin University of Traditional Chinese Medicine) and the voucher specimens were deposited at Tianjin University of Traditional Chinese Medicine,Tianjin, China. Columbianetin acetate (purity>98%) was isolated from APR in our laboratory. Deionized water was purified with a Milli-Q Academic ultra-pure water system (Millipore, Milford, MA, USA).

2.2. Apparatus and conditions

Chromatographic separation was carried out on an Agilent 1200 series liquid chromatographic system (Agilent Technologies, Santa Clara, CA, USA) equipped with G1322A degasser, G1312A Bin Pump, G1367B autosampler, G1316A thermostatic column compartment and an Agilent Eclipse Plus C18 (4.6×100 mm, 1.8μm) column with a security guard C18 (2.1 mm×12.5 mm, 5μm) column. The column oven temperature was set at 20□. According to our previous study, acetonitrile (solvent system A) and water containing 1 mmol L^{-1} ammonium acetate (solvent system B) was selected as mobile phase with a gradient elution of 40%A at 0-5 min, 40-70%A at 5-10 min, 70-75%A at 10-11 min, 75-90%A at 11-19 min,

90%A at 19-23 min [22]. The re-equilibration time of gradient elution was 5 min. The flow rate was set at 0.3 mL min^{-1} and the injection volume was 5 μL.

Mass data were acquired from an API 3200 tandem mass spectroscopy (Concord, Ontario, Canada) and performed with Analyst 1.4.2 software (AB MDS Sciex). The components were detected and quantified by electrospray ionization (ESI) in positive ion mode and multiple-reaction monitoring (MRM) mode, respectively. Spray voltage was at +5500V. Turbo spray temperature was set at 400□. The collision gas, nebulizer gas (gas 1), auxiliary gas (gas 2) and curtain gas were optimized at 5, 6, 40 psi and 25 psi respectively. The precursor-to-product ion pairs, dwell time, declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP) for columbianetin, columbianetin acetate and warfarin (IS) were set according to the previous studies [22- 23]

2.3. Preparation of APR extract and isolation of columbianetin acetate

APR was air-dried, cut into pieces and soaked in 75% ethanol for 8 h in a separation funnel. The solvent level was maintained above the medicinal material even after swelling. The ratio of material to solvent was 1:8. After percolation, the ethanol extract was concentrated by rotary evaporator under reduced pressure and dried out in a vacuum oven to get the APR extract. APR extract was dissolved in water and extracted with ethyl acetate for three times. HPLC showed that all the columbianetin acetate was in the ethyl acetate phase after triple extractions. Six fractions were obtained from the ethyl acetate phase of APR extract by normal-phase silica column chromatography (petroleum ether-ethyl acetate (4:1, v/v)). Fraction 5

was columbianetin acetate. Its purity was more than 98%.

2.4. Quantitative determination of columbianetin acetate in APR extract

The concentration of columbianetin acetate in APR extract was determined by HPLC-UV according to our previous studies [14]. The powdered APR extract (102 mg) were accurately weighed in triplicate. The sample was extracted with 10 ml of methanol by using an ultrasonic bath for 40min and then cooled at roo m temperature. Methanol was added to compensate for the lost weight. The solution was centrifuged at 14000 rpm for 10 min at 4℃. The supernatant was transferred into vials for injection. Quantitative determination of columbianetin acetate in APR extract was conducted on HPLC –UV (Agilent 1200 series liquid chromatographic system).

2.5. Preparation of stock and working solutions

Stock solutions of columbianetin and columbianetin acetate were both dissolved in methanol to a concentration of 1 mg mL^{-1}, respectively. Internal standard warfarin solution was prepared in methanol at a concentration of 100 ng mL^{-1}. Appropriate aliquots of stock solutions of columbianetin and columbianetin acetate were mixed to prepare a mixed stock solution. To prepare the standard solutions for calibration and quality control samples, the mixed stock solution was serially diluted with methanol to obtain working stock solutions of final concentration of 0.5, 1, 2, 5, 10, 50, 100, 500, 1000, 2000, 5000 ng mL^{-1}. All the solutions were stored at 4□ and brought to room temperature before use.

2.6. Preparation of samples and quality control samples

100 μL plasma samples and 10 μL internal standard (IS) solutions were added into

a 1.5 mL eppendorf tube. Samples were extracted with 1000 μL ethyl acetate, vortexed for 3 min to mix sufficiently and then centrifuged at 14,000 rpm for 10 min at 4□. The supernatants were transferred into another eppendorf tube and dried out under a nitrogen stream. The residue was redissolved in 100 μL methanol, oscillated ultrasonically at a work frequency of 40 kHz, and then centrifuged again at 14,000 rpm for 10 min at 4℃. Subsequently, 5 μL aliquot of the supernatants were transferred into vials for injection.

Quality control (QC) samples at low, medium and high concentration (2, 100 and 1000 ng mL^{-1}) were prepared by spiking appropriate standard solutions of columbianetin and columbianetin acetate into blank plasma to the required plasma concentrations following the same preparation and operation method described above.

2.7. Method validation

2.7.1. Specificity and sensitivity

The chromatography of six different batches of blank rat plasma samples and the corresponding spiked plasma were compared in order to test the specificity. For determination of columbianetin and columbianetin acetate, the lower limit of quantification (LLOQ) was defined as the lowest concentration in the calibration curve. The signal to noise ratio (S/N) at this concentration was larger than 5 while accuracy ranged from 80 to 120% and the relative standard deviation (RSD) (n=6) was within 20% (Guidance for industry: bioanalytical method validation, U.S.Department of Health and Human Services, 2001)[24].

2.7.2. Linearity

Calibration curve samples were prepared by spiking blank rat plasma with working stock solutions prepared as described above. Using linear regression analysis, calibration curves were plotted by the peak-area ratios (y) of analyte against internal standard versus the nominal concentration (x).

2.7.3. Precision and accuracy

The validation of intra-assay precision and accuracy of the method were evaluated by determination of LLOQ samples and QC samples (n=6) at three concentrations (1, 2, 100, 1000 ng mL^{-1}). For the validation of inter-assay precision and accuracy, LLOQ, low, medium and high QC samples from three assays analysed were evaluated. Precision was expressed by relative standard deviation (RSD) and accuracy was calculated as a ratio of mean measured concentration and nominal concentration.

2.7.4. Recovery and matrix effect

The recoveries for two analytes in rats plasma were determined in six replicates of three levels of QC samples (2, 100, 1000 ng mL^{-1}). They were calculated as the percentage of analyte peak area from extracted QC plasma to mean peak area from extracted blank plasma spiked with the neat solutions. The matrix effects for two analytes were determined using extracted blank rats plasma samples spiked with two analytes at three QCs concentrations (2, 100, 1000 ng mL^{-1}). Matrix effect was determined as the percentages of analyte peak areas from extracted blank plasma spiked with the two analyte solutions to the mean peak area of the analyte solutions at the same concentration prepared in the HPLC eluent.

2.7.5. Stability experiments

Stability experiments were conducted to confirm the stability under the conditions that samples might be exposed to during storage and handling. The stability of freeze/thaw cycles, long-term and in autosampler for 24 h were all performed at three concentrations (low, medium, high) of the QC samples using six replicates. For freeze/thaw cycles test, QC samples were frozen at -20℃ and thawed at room temperature for a period of 12 h for three times. For long-term stability test, QC samples were frozen at -20℃ for two weeks and were thawed at room temperature before injection. The stability of processed QC samples kept in autosampler for 24 h was also investigated.

Experiments were also carried out to evaluate the stability of columbianetin acetate and columbianetin alone in rat plasma, respectively. The interconversion between these two compounds, which may exist during the process of analysis, was also been tested. Working stock solutions and QC samples for columbianetin and columbianetin acetate alone were respectively prepared as described above.

2.8. Excretion of columbianetin acetate and columbianetin as its metabolite in rat urine and feces

Ten rats were housed in stainless steel metabolic cages provided with urine–feces separators. The rats were fasted overnight but had free access to water. The rats were orally administrated with columbianetin acetate which had been dissolved in 0.5% CMC-Na at a dosage of 5 mg kg^{-1} and then placed in metabolic cages that allowed for the separate collection of urine and feces. Urine and feces samples for each rat were collected at pre-dose (0 h) and at different time intervals (0-4, 4-8, 8-12, 12-24, 24-36,

36-48, 48-60, and 60-72 h). After the volumes of urine obtained were measured, urine samples collected were centrifuged at 14000 rpm for 10 min at 4℃, the supernatants of urine samples and feces samples were stored at -20℃ until analysis. Preparation of urine samples was the same as the preparation of plasma samples as described above. feces samples were dried out in drying oven at 40℃. After the weights of feces obtained were measured, they were crushed and homogenized by mortar. The preparation method of feces samples was similar to that of herbal sample preparation according to our previous study [25].The procedure was briefly as follows. 10 mg of feces powder and 1 mL of 70% methanol was added into 1.5 mL polythene tubes, mixed sufficiently for 3 min by vortex and ultrasonic extracted for 30 min. Then feces samples were centrifuged at 14000 rpm for 10 min at 4□. 90 μL of the supernatant and 10 μL of IS were mixed together and transferred into vials for analysis by LC-MS/MS.

2.9. Pharmacokinetic study in rats

Male Sprague-Dawley rats (230 ± 20 g weight) were maintained in a rodents feeding room under a light/dark cycle of 12/12 h for 7 days before use. Food and water were given ad libitum except for 12 h before the experiment. Animal welfare and experimental procedures were under the guidance for the care and use of laboratory animals and Ethics Review Committee of Tianjin University of Traditional Chinese Medicine. Columbianetin acetate for the study of intravenous administration was grinded with gum arabic (1:15, w/w) and dissolved in normal saline to a concentration of 5 mg mL^{-1}. For the study of oral administration, columbianetin

acetate and APR extract was dissolved in 0.5% carboxymethyl cellulose sodium solution (CMC-Na). The rats were randomly divided into five groups. The first group was given columbianetin acetate intravenously at a dose of 5 mg kg^{-1}(i.v. 5mg group). Two of these groups were orally administrated with columbianetin acetate at a dose of 5 mg kg^{-1} (i.g. 5 mg kg^{-1} columbianetin acetate) and APR extract at a corresponding dose (i.g. 5 mg kg^{-1} columbianetin acetate in extract), respectively. Another two group were orally administrated with columbianetin acetate at a dose of 10 mg kg^{-1} (i.g. 10 mg kg^{-1} columbianetin acetate) and APR extract spiked with columbianetin acetate at a corresponding dose of 10 mg kg^{-1} (i.g. 10 mg kg^{-1}APR adding columbianetin acetate), respectively. Blood samples (250 μL) were collected from sub-orbital vein into 1.5 mL polythene tubes, which had been heparinized to prevent blood coagulation, at 0.033, 0.083, 0.167, 0.33, 0.5, 0.75, 1, 2, 3, 4, 5, 6, 8 h after intravenous administration, and at 0.033, 0.083, 0.167, 0.33, 0.5, 0.75, 1, 2, 4, 5, 6, 8, 12, 24 h after oral administration, respectively. Blood samples were immediately centrifuged at 4000 rpm for 10 min at 4℃ to get plasma after collection. Plasma was transferred into non-heparinized tubes and stored at -20℃ until analysis.

2.10. Chemical hydrolysis analysis

The samples of APR extract, columbianetin acetate and pure columbianetin were dissolved in 3 mL methanol. After completely dissolved by ultrasonic, 2 mL of diluted hydrochloric acid was added into the reaction system, adequately mixed by vortex and then placed in water bath at 37□, sufficiently reacted for 2 h. After reaction, solutions were centrifuged at 14000 rpm for 10 min at 4℃, the supernatants were transferred

into vials for analysis by ultra-high performance liquid chromatography) equipped with PDA detector(UHPLC-PDA). In this study, standard reaction solutions (1×) was prepared by diluting 234 mL of concentrated hydrochloric acid(12mol L^{-1}) into 1000 mL distilled water. To investigate the chemical hydrolyzation in different acidic conditions, eight different concentrations of hydrolysis solutions (standard reaction solutions (1×), 2 folds (2×), 4 folds (4×), 6 folds (6×), 8 folds (8×), 12 folds (12×), 16 folds (16×) and 20 folds (20×) of standard reaction solutions) were selected. The method of quantitative analysis by UHPLC-PDA was the same as our previous study [26]. All the chemical hydrolyzation experiments were carried out in triplicates; results were shown by means ± standard deviation.

2.11. Data analysis

"Drug and Statistics 1.0" program (DAS 1.0) (Medical College of Wannan, China) was employed for the purpose of analyzing the pharmacokinetic parameters after intravenous administration of columbianetin acetate and oral administration of columbianetin acetate and APR extract, respectively. AIC (Akaike information criterion) was used to judge compartment model of columbianetin acetate.

3. Results and discussion

3.1. Determination of the concentration of columbianetin acetate in APR extract

. Standards of columbianetin acetate with concentration of 5, 10, 25, 50, 125, 150 μg mL^{-1} were detected by HPLC-UV-detector and a standard curve was drawn. APR extract was dissolved in methanol, and then detected by HPLC. The standard curve equation was y = 0.0672x - 0.291 (y: peak-area ratios, x: concentration). The results

indicate that in order to give the same dose of 5 mg kg^{-1} columbianetin acetate, the dose of corresponding APR extract should be 0.72 g kg^{-1}.

3.2 Optimization of analytical method

It has already been confirmed that liquid–liquid extraction with ethyl acetate is suited to extract coumarins such as columbianetin acetate and columbianetin with excellent specificity and reproducibility in our previous studies [22]. Warfarin (structure shown in Fig.1) was selected as internal standard (IS) in this study. Belonging to coumarins, warfarin, columbianetin acetate and columbianetin have similar chemical structural formula. Until now, researches show that there is no warfarin in APR [22]. Thus, taking warfarin as IS will not affect the results especially when APR extract is administered orally. Furthermore, method validation has proven that warfarin has no interference from the endogenous matrix, and these three coumarins were easily quantified by HPLC-MS/MS without cross interference. Thus, warfarin was an excellent IS for quantifying the concentration of columbianetin acetate and columbianetin in rat plasma. Liquid–liquid extraction with ethyl acetate was selected to extract columbianetin acetate and columbianetin from rat plasma samples.

3.3. Method validation

3.3.1. Specificity and sensitivity

The representative chromatograms of blank plasma and blank plasma spiked with columbianetin, columbianetin acetate and warfarin (IS), respectively, are shown in Fig.2. The retention time of columbianetin was about 8.06 min, columbianetin acetate

was 16.28 min and IS was 15.43 min. This suggests that no interferences from endogenous plasma substances or metabolites were observed and good separation of the analytes was achieved.

3.3.2. Calibration curve and lower limits of quantification

The plasma calibration curves for both columbianetin acetate and columbianetin performed a reliable reproducibility over the range of 1-5000 ng mL^{-1}. The regression equation obtained for columbianetin acetate and columbianetin were y=0.0173x+0.0523 and y=0.104x+0.186, using weighing factor ($1/x^2$), respectively. The lower limit of quantification (LLOQ) for determination of columbianetin and columbianetin acetate in plasma were both 1 ng mL^{-1}. The calibration curves of the stability experiments of columbianetin acetate and columbianetin alone in plasma were y=0.00563x+0.00778 (r=0.9978) and y=0.00327x+0.00286 (r=0.9988), using weighing factor ($1/x^2$), respectively. For the quantitative analysis by UHPLC, the calibration curves of columbianetin acetate and columbianetin were y=15792.776-440.9404 (r=0.9996) and y=17162.017-393.88545 (r=0.9999), using weighing factor ($1/x^2$), respectively.

3.3.3. Precision and accuracy

The intra-assay and inter-assay precision and accuracy of the method were summarized in **Table 1** by analyzing LLOQ samples and QC samples at three concentrations in six replicates. For the results of QC samples, the intra-assay precision was less than 11.6% for columbianetin, 10.9% for columbianetin acetate while the inter-assay precision was within 13.2% for columbianetin and within 11.9%

for columbianetin acetate. For the results of LLOQ samples, the intra-assay precision was 8.14% and 19.0% for columbianetin and columbianetin acetate, respectively. The inter-assay precision was 6.25% for columbianetin and 16.7% for columbianetin acetate. The intra-assay accuracy ranged from 90.2% to 99.0% and the inter-assay accuracy ranged from 89.6% to 100% for columbianetin and columbianetin acetate. Following the USFDA guidelines, all the intra- and inter-assay values for both columbianetin and columbianetin acetate were found to be within the acceptable variable limits. According to these results, the method was confirmed to be precise and accurate for determination of columbianetin and columbianetin acetate in rat plasma.

3.3.4. Recovery and matrix effects

The extraction recoveries were 114%, 101% and 110% for columbianetin and 103%, 98.5%, 93.4% for columbianetin acetate QC samples at the concentrations of 2, 100 and 1000 ng mL^{-1}, respectively. The corresponding RSDs were 12.8%, 9.98% and 4.97% for columbianetin and 8.61%, 2.96% and 3.84% for columbianetin acetate, respectively (**Table 2**). As shown in Table 2, the matrix effects of columbianetin and columbianetin acetate ranged from 101% to 119%, 104% to 112% for all levels of QC samples, respectively. Based on the results, the method of extraction with ethyl acetate could be proved to be precise and feasible for columbianetin and columbianetin acetate from plasma samples and conclusion could be drawn that endogenous substances have little influence on the determination of columbianetin and columbianetin acetate in rat plasma. These results were consistent with our

previous study.

3.3.5. Stability

The results of stability experiments of freeze/thaw cycles, stability for two weeks and in autosampler for 24 h, for columbianetin and columbianetin acetate, both together and alone in plasma, are shown in **Table 3A and Table 3B**. Even though it had been found that (shown in Table 3C) 0.49 ± 0.07% at high level (1000 ng mL^{-1}) and 0.05 ± 0.01% at medium level (100 ng mL^{-1}) of columbianetin acetate were converted into columbianetin in the experiments of stability for two weeks for columbianetin acetate alone in plasma and this phenomenon of conversion also occurred in the experiments of freeze/thaw cycles at high level (1000 ng mL^{-1}) that 2.07 ± 0.10% of columbianetin acetate changed, the results were all within the acceptable variable limits, indicating that columbianetin and columbianetin acetate in rat plasma were stable after freeze/thaw cycles for three times, after being frozen for two weeks and after being kept in autosampler for 24 h. The results suggested that samples should be detected in two weeks and the storage and handling of samples have no significant effect on the results of the analysis during the experimental process.

3.4. Pharmacokinetic studies

The developed LC-MS/MS method was successfully applied to the pharmacokinetic study of columbianetin acetate and columbianetin in rat plasma following intravenous and oral administration of pure columbianetin acetate and oral administration of APR extract.

3.4.1. Pharmacokinetics of columbianetin and columbianetin acetate in rats after intravenous administration of pure columbianetin acetate

The mean plasma concentration–time profiles of columbianetin acetate after intravenous administration of columbianetin acetate at a dose of 5 mg kg^{-1} to rats are shown in **Fig. 3**. According to AIC comparison and model diagnostics, the model with lower AIC value should be a priority. The two-compartment model (AIC value = 103.2) should be preferred over one-compartment model (AIC value = 122.3), obviously. Relevant pharmacokinetic parameters are listed in **Table 4**. Columbianetin acetate was distributed to tissues rapidly based on the distribution half-life ($T_{1/2\alpha}$) value of 3 min. Taking the average steady-state volume of distribution (V) and the value of V_1 into comparison, the former (0.0076 ± 0.0062 L) was 5.42 fold of the latter (0.0014 ± 0.0005 L). These suggested that columbianetin acetate was distributed rapidly and widely in rats. Elimination half-life ($T_{1/2\beta}$) was valued as 0.8302 ± 0.7805 h, indicating a rapid elimination for columbianetin acetate from plasma in rats. Total clearance (CL) of columbianetin acetate was assessed to be 0.0069 ± 0.0014 L h^{-1}.

When detecting columbianetin acetate, the data of columbianetin was also collected by LC-MS after intravenous administration of columbianetin acetate. The mean plasma concentration–time profiles of columbianetin are shown in **Fig. 3**. It could be concluded that columbianetin acetate was metabolized to columbianetin in vivo. For columbianetin, the two-compartment model (AIC value = 74.38) was lower than one-compartment model (AIC value = 97.06). Thus, two-compartment model was selected for columbianetin. The pharmacokinetic parameters are also listed in **Table 4**

After intravenous administration of pure columbianetin acetate, the AUC(0-t_n) values of columbianetin acetate and columbianetin were 710 ± 149 μg $L^{-1}$and 145 ± 48 μg L^{-1}, respectively. According to the chemical structures of columbianetin acetate and columbianetin shown in Fig.1, one molecule of columbianetin metabolite comes from one molecule of columbianetin acetate. Thus, the amount of columbianetin acetate that metabolized into columbianetin could be calculated. It was found that approximately 23.91% of columbianetin acetate is metabolized into columbianetin by the liver after intravenous administration of columbianetin acetate. Combining with the preliminary pharmacological experiments, it suggests that columbianetin is one of the main metabolites of columbianetin acetate which play a role in its efficacy.

3.4.2. Pharmacokinetics of columbianetin and columbianetin acetate in rats after oral administration of pure columbianetin acetate

In accordance with AIC comparison and model diagnostics, one-compartment model (AIC value = 57.85) was more suitable than two-compartment model (AIC value = 58.85). Thus, the one compartment model was selected to describe the pharmacokinetic profile of columbianetin acetate after oral administration of columbianetin acetate. The mean plasma concentration–time profiles of columbianetin acetate and columbianetin are shown in **Fig. 5** after oral administration of columbianetin acetate at a dose of 5 mg $kg^{-1}$and 10 mg kg^{-1} to rats. Some relevant pharmacokinetic parameters are listed in **Table 5.** After oral administration of columbianetin acetate, columbianetin acetate was rapidly absorbed into the blood from the rat gastrointestinal tract. Columbianetin acetate was detected in the plasma

from the first blood sampling time (2 min) and rapidly reached Tmax (0.53–0.56 h) for two oral doses studied. The elimination time of columbianetin acetate is short because $T_{1/2\alpha}$ value was 3.44 ± 0.71 h. The $AUC_{(0\text{-}tn)}$ of columbianetin acetate at doses of 5 and 10 mg kg^{-1} were 49.89 ± 31.21 and 122.2 ± 34.1μg L^{-1} h^{-1}. It was noted that the dose-normalized AUC values of columbianetin acetate (based on 5 mg kg^{-1}) were proportional to the doses studied.

After oral administration of columbianetin acetate to rats, columbianetin had high plasma concentration, its T_{max} was 0.52 ± 0.35h, C_{max} was 25.65 ± 9.17μg L^{-1} and $T_{1/2\alpha}$ was 1.18 ± 0.67 h, respectively. Based on these results, it can be seen that a large amount of columbianetin acetate was transformed into columbianetin and its metabolite columbianetin can be eliminated rapidly from the body. Comparing the percentage of columbianetin acetate ($AUC_{(0\text{-}tn)}$ values of after oral administration(49.89 ± 31.21) to $AUC_{(0\text{-}tn)}$ values after intravenous administration(710 ± 149)) and that of columbianetin (51.58 ± 20.08/145 ± 48) at the same dose of 5 mg kg^{-1} (**Table 4** and **Table 5**), it was worth noting that the former was lower than the latter. The mean Tmax for columbianetin acetate and columbianetin was 0.56 h and 0.52 h, respectively. That is to say, the maximum concentration of the metabolite was observed before the parent. According to the phenomenon that columbianetin acetate could be converted into columbianetin in acidic solution. Columbianetin has a high bioavailability in our previous studies and rapidly reached Tmax (11–43 min) for all three oral doses studied [27].it was demonstrated that an amount of columbianetin acetate was be hydrolyzed into columbianetin in the stomach under acidic conditions

so that the maximum concentration of the metabolite with high plasma concentration was observed before the parent. The reasons for this result still need further study.

3.4.3. Pharmacokinetics of columbianetin and columbianetin acetate in rats after oral administration of APR extract

As one of the main components in APR, columbianetin acetate has a high concentration in APR extract. In order to investigate the difference between oral administration of columbianetin acetate and APR extract, APR extract containing corresponding doses of columbianetin acetate (5 mg Kg^{-1}) was given to rats orally. Columbianetin acetate and columbianetin were collected at the same time, and mean plasma concentration–time profiles of columbianetin acetate and columbianetin were plotted in **Fig. 5.** Main pharmacokinetic parameters are exhibited in **Table 5**. After oral administration of APR extract, the T_{max}, C_{max} and $AUC_{(0-tn)}$ value for columbianetin acetate was 0.49 ± 0.15 h, 34.15 ± 7.99 μg L^{-1} and 78.06 ± 15.75 μg L^{-1} h^{-1}, respectively. There were no significant differences between plasma concentrations of columbianetin acetate after oral administration of pure columbianetin acetate and that of APR extract. The C_{max} and $AUC_{(0-tn)}$ value of columbianetin was 73.35 ± 48.54 μg L^{-1} and 98.08 ± 56.26 μg L^{-1} h^{-1}, respectively. Comparing with oral administration of columbianetin acetate with corresponding dose, the C_{max} and $AUC_{(0-tn)}$ value of columbianetin after oral administration of APR extract were increased. This phenomenon indicated that other co-existing ingredients in APR extract could increase the concentration of columbianetin in plasma. There are two possibilities to explain these results. First of all was that other derivatives of columbianetin, in

particular glycosides, are present in the extract and they are transformed to the molecules of interest in the organism. In this study, the hydrolysis analysis of the extract and pure components were selected to evaluate this problem. In order to make other derivatives of columbianetin be fully hydrolyzed into columbianetin, the different concentration of hydrochloric acid were used to hydrolyze the APR extract and corresponding concentration of pure columbianetin acetate and columbianetin. It was found that columbianetin is stable in the different concentrations of hydrochloric acid. Columbianetin acetate could be hydrolyzed into columbianetin. The APR extract could also be hydrolyzed into columbianetin. As shown in figure 8, the hydrolysis percentages of columbianetin acetate were closely coincident. It was summarized that there are no influence of other co-existing ingredients in APR extract on transformation of columbianetin acetate into columbianetin. From **figure 7,** the concentrations of columbianetin increased from the hydrolysis of APR extract were higher than those from the hydrolysis of pure columbianetin acetate at all different concentrations of hydrochloric acid. It could be concluded that other derivatives of columbianetin could be hydrolyzed into columbianetin. According to our previous results [26], derivatives of columbianetin concluded columbianadin and columbianetin-β-D-glucopyranoside. It was also found that the concentration of columbianadin in APR extract was increased when concentrations of hydrochloric acid was increased. Another reason is that co-existing ingredients in APR extract could change of pharmacokinetic behave of columbianetin acetate. In order to confirm the enhancement of absorption or influence on metabolism alteration of the

pharmacokinetic in the APR extract, additional pharmacokinetic were performed adding known amount of purified columbianetin acetate in the extract (corresponding doses of columbianetin acetate (10 mg Kg^{-1}). It was found that there were no significant differences between plasma concentrations of columbianetin acetate and its metabolite columbianetin after between oral administration of pure columbianetin acetate at dose of 10 mg Kg^{-1} and that of APR extract adding known amount of purified columbianetin acetate (10 mg Kg^{-1}). Based on these results, pharmacokinetic behave of columbianetin acetate which was added in the extract is enhanced proportionally like those of columbianetin acetate which was added into the purified samples. Therefore, other components in APR extract not change of pharmacokinetic behave of columbianetin acetate, which is not main reason why other co-existing ingredients in APR extract could increase the concentration of columbianetin in plasma. Based on the above results, columbianetin-β-D-glucopyranoside, one of the derivatives of columbianetin in APR extract might be transformed to columbianetin so that APR extract could increase the concentration of columbianetin in plasma

3.5 Oral bioavailability of columbianetin acetate in rats after administration

As pharmacokinetic parameters shown in Table 7, oral bioavailability of pure columbianetin acetate and columbianetin acetate in APR extract were calculated. In comparison with the $AUC_{(0\text{-}tn)}$ value of pure columbianetin acetate by oral administration and intravenous administration, the absolute bioavailability of columbianetin acetate were 7.0 ± 4.3% and 11 ± 2% in pure form and APR extract, respectively.

3.6. Excretion study of columbianetin acetate and its metabolite columbianetin in rat urine and feces

Characteristics of cumulative urinary excretion of columbianetin acetate were determined. After oral administration of 5 mg kg^{-1} columbianetin acetate to rats, both columbianetin acetate and columbianetin were found in urine and feces. It is demonstrated that columbianetin acetate was metabolized to columbianetin in vivo. columbianetin acetate and columbianetin could be detected in rat urine until 72 h after dosing. From 12 to 36 h, columbianetin acetate and columbianetin levels in rat urine increased quickly. As shown in **Fig 3**, cumulative excretion of columbianetin acetate in urine accounted for 0.0109 ± 0.0067% of total dosage of columbianetin acetate in its original form at 72 h after administration. Columbianetin as one of metabolites of columbianetin acetate was excreted in the urine was 0.0044 ± 0.0026% of total dosage of columbianetin acetate. The above results indicated that the majority of dosed columbianetin acetate might have been metabolized in rats. Columbianetin acetate and columbianetin were also determined in feces samples after dosing. The cumulative excretion of columbianetin acetate showed a continuous increase until 36 h. The maximum excretion of columbianetin acetate and columbianetin into feces was observed from 12 to 24 h. The cumulative amounts of columbianetin acetate and columbianetin present 9.32 ± 6.63% and 2.81 ± 1.15% of the total dose, which indicate that the columbianetin acetate was mainly excreted in the feces. In the present study, only columbianetin acetate and its metabolite columbianetin were determined in rat urine and feces. The other metabolites of columbianetin acetate need further

study.

4. Conclusion

A sensitive, accurate and precise method has been established for simultaneous determination of the concentration of columbianetin acetate and columbianetin in rat plasma by LC-MS/MS. The pharmacokinetic profiles of columbianetin acetate and columbianetin after intravenous and oral administration of pure columbianetin acetate, and oral administration of APR extract to rats were obtained. The results showed that columbianetin acetate could be metabolized into columbianetin *in vivo*. The oral bioavailability of columbianetin acetate in rats after administration of pure columbianetin acetate was 7.0 ± 4.3%. By comparing pharmacokinetic parameters of columbianetin after oral administration of pure columbianetin acetate with that of APR extract, the results suggest that other co-existing ingredients in APR extract could promote the concentration of columbianetin in rat plamas. Cumulative excretion of columbianetin acetate in urine accounted for 0.0109 ± 0.0067% of total dosage. The cumulative amounts of columbianetin acetate in the feces present 9.32 ± 6.63% of the total dose. Columbianetin acetate was mainly excreted in the feces. These results might be helpful for further in vivo study and clinical application of columbianetin acetate and APR extract in clinic.

Acknowledgments

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Figure legends:

Fig.1 Chemical structures of columbianetin, columbianetin acetate and warfarin (IS).

Fig.2 Representative chromatogram of (A) blank rat plasma,(B) blank rat plasma spiked with standard compounds at LLOQ and (C) real sample after administration of columbianetin acetate

Fig.3 Time cumulative excretion percentage of columbianetin acetate and its metabolite columbianetin (A) in urine and (B) in feces; Time -cumulative excretion percentage of columbianetin acetate and its metabolite columbianetin (C) in urine and (D) in feces.

Fig.4 The mean plasma concentration-time profiles of columbianetin acetate and columbianetin after intravenous administration of pure columbianetin acetate..

Fig.5 The mean plasma concentration-time profiles of (A) columbianetin acetate of i.g. 5 mg kg^{-1} columbianetin acetate and 5 mg kg^{-1} columbianetin acetate in extract (B) columbianetin acetate of i.g. 10 columbianetin acetate and 10 mg kg^{-1} columbianetin acetate in extract ; (C) columbianetin of of i.g. 5 mg kg^{-1} columbianetin acetate and 5 mg kg^{-1} columbianetin acetate in extract (D) columbianetin of of i.g. 10 mg kg^{-1} columbianetin acetate and 10 mg kg^{-1} columbianetin acetate in extract .

Fig 6. Relationship between percentage of actual increment of columbianetin and various folds of acidity of hydrolysis solution

Fig.7 Relationship between percentage of decrement of columbianetin acetate and various folds of acidity of hydrolysis solution

Table.1 **Intra-assay, inter-assay accuracy and precision of columbianetin acetate and columbianetin of the assay (n=6).**

	Concentration (ng mL^{-1})	Intra-assay		Inter-assay	
		Accuracy(%)	RSD(%)	Accuracy(%)	RSD(%)
Columbianetin	1	106	8.14	107	6.25
	2	99.0	11.6	92.5	13.2
	100	92.1	6.13	93.4	5.79
	1000	97.4	8.13	100	7.71
Columbianetin acetate	1	101	19.0	99.2	16.7
	2	90.0	8.32	90.3	11.9
	100	90.2	10.9	89.6	7.43
	1000	93.5	5.31	93.0	6.10

Table2**Recovery and matrix effects of columbianetin acetate and columbianetin of the assay (n=6).**

	Concentration (ng mL^{-1})	Recovery		Matrix effects	
		Mean (%)	RSD (%)	Mean (%)	RSD (%)
Columbianetin	2	114	12.8	119	9.94
	100	101	9.98	101	8.41
	1000	110	4.97	103	2.65
Columbianetin acetate	2	103	8.61	112	14.5
	100	98.5	2.96	113	10.9
	1000	93.4	3.84	104	3.26

Table.3A **Stability of columbianetin acetate and columbianetin together in the plasma (n=6).**

	Concentration ($ng\ mL^{-1}$)	Freeze-thaw cycles		Stability for two weeks		Autosampler for 24h	
		Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
Columbianetin	2	84.8	15.0	113	12.8	94.5	11.8
	100	92.8	9.54	97.5	9.11	96.7	7.14
	1000	114	6.95	113	7.37	104	8.60
Columbianetin acetate	2	83.8	8.38	83.7	12.3	90.0	8.32
	100	106	12.8	102	6.42	87.1	4.75
	1000	100	6.36	99.8	8.90	86.2	10.1

Table.3B **Stability of columbianetin acetate and columbianetin alone in the plasma (n=6).**

	Concentration ($ng\ mL^{-1}$)	Freeze-thaw cycles		Stability for two weeks		Autosampler for 24h	
		Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
Columbianetin	2	115	12.6	106	8.83	110	9.16
	100	103	4.72	103	3.55	110	3.96
	1000	104	4.29	104	4.42	106	2.68
Columbianetin acetate	2	111	10.2	115	9.34	109	13.2
	100	107	2.92	103	6.01	105	4.41
	1000	109	2.11	95.5	6.08	109	2.99

Table.3C

Columbianetin acetate that have been converted to columbianetin during stability experiments

	Concentration ($ng\ mL^{-1}$)	Freeze-thaw cycles	Stability for two weeks
Columbianetin acetate	2	-	-
	100	-	0.05 ± 0.01 %
	1000	2.07 ± 0.10 %	0.49 ± 0.07 %

Table 4

Pharmacokinetics of columbianetin and columbianetin acetate in rats after intravenous administration of columbianetin acetate

Parameters	Columbianetin acetate	Columbianetin as the metabolite of columbianetin acetate
T_{max}(h)	0.03 ± 0.00	0.03 ± 0.00
C_{max}(μg L^{-1})	2283 ± 826	539 ± 263
k_{10}(h^{-1})	5.53 ± 3.38	10.90 ± 6.36
k_{12}(h^{-1})	10.71 ± 4.60	26.77 ± 11.79
k_{21}(h^{-1})	4.84 ± 2.94	6.14 ± 2.99
V_1(L)	0.0014 ± 0.0005	0.0047 ± 0.0034
V(L)	0.0076 ± 0.0062	0.0312 ± 0.0184
$T_{1/2\alpha}$(h)	0.0407 ± 0.0151	0.0195 ± 0.0093
$T_{1/2\beta}$(h)	0.8302 ± 0.7805	0.5824 ± 0.3370
CL(L h^{-1})	0.0069 ± 0.0014	0.0359 ± 0.0095
AUC(0-t_n)(μg L^{-1} h)	710 ± 149	145 ± 48
AUC(0-∞)(μg L^{-1} h)	757 ± 169	148 ± 47

Table.5**Pharmacokinetics of columbianetin and columbianetin acetate in rats after oral administration of columbianetin acetate and APR extract**

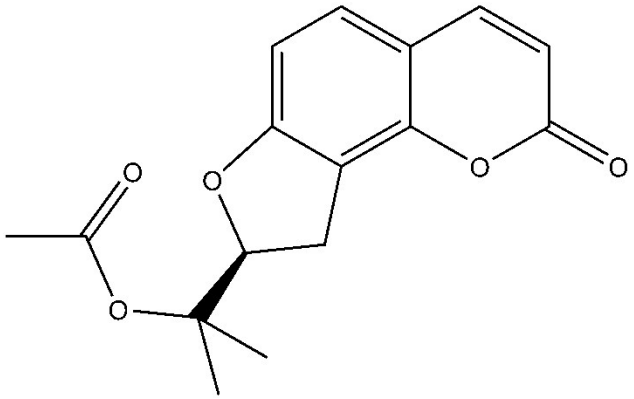
	Oral administration of columbianetin acetate (5 mg kg^{-1})		Oral administration of APR extract(5 mg kg^{-1})		Oral administration of columbianetin acetate (10 mg kg^{-1})		Oral administration of APR extract and columbianetin acetate (10 mg kg^{-1})	
Parameters	Columbianetin acetate	Columbianetin as metabolite of columbianetin acetate	Columbianetin acetate	Columbianetin	Columbianetin acetate	Columbianetin as metabolite of columbianetin acetate	Columbianetin acetate	Columbianetin
T_{max}(h)	0.56 ± 0.39	0.52 ± 0.35	0.49 ± 0.15	0.66 ± 0.23	0.53 ± 0.39	0.52 ± 0.31	0.38 ± 0.26	0.59 ± 0.38
C_{max}(μg L^{-1})	25.34 ± 16.96	25.65 ± 9.17	34.15 ± 7.99	73.35 ± 48.54	33.28 ± 4.35	31.86 ± 11.93[c]	22.68 ± 12.57	19.05 ± 14.68
k_a(h^{-1})	23.49 ± 28.62	12.05 ± 17.59	6.27 ± 2.14	14.49 ± 8.85	41.31 ± 36.81	12.39 ± 3.77[d]	32.91 ± 32.11	35.92 ± 36.70
k_e(h^{-1})	0.19 ± 0.06[a]	0.78 ± 0.58	0.39 ± 0.17	0.71± 0.45	0.30 ± 0.40	0.46 ± 0.25[d]	0.19 ± 0.11	0.15 ± 0.08
V/F(L)	0.26 ± 0.14[a]	0.25 ± 0.25	0.13 ± 0.04	0.11 ± 0.15	0.39 ± 0.14[d]	0.47 ± 0.16	1.17 ± 0.55	0.79 ± 0.45
$T_{1/2ka}$(h)	0.038 ± 0.052[a]	0.15 ± 0.18	0.13 ± 0.06	0.10 ± 0.12	0.031 ± 0.045	0.044 ± 0.042	0.033 ± 0.047	0.030 ± 0.039
$T_{1/2\alpha}$(h)	3.44 ± 0.71[a]	1.18 ± 0.67	2.99 ± 0.78	1.30 ± 0.63	2.43 ± 1.08	1.90 ± 0.88[c]	3.63 ± 1.53	3.83 ± 0.88
Lag time(h)	0.030 ± 0.039	0.036 ± 0.045	0.044 ± 0.035	0.071 ± 0.031	0.046 ± 0.038	0.033 ± 0.036	0.020 ± 0.030	0.067 ± 0.059
AUC(0-t_n)(μg L^{-1} h^{-1})	49.89 ± 31.21	51.58 ± 20.08	78.06 ± 15.75	98.08 ± 56.26	122.2 ± 34.1	82.57 ± 24.99	89.51 ± 57.89	102.4 ± 52.2
AUC(0-∞)(μg L^{-1} h^{-1})	56.73± 37.35	54.29 ± 24.18	96.30 ± 33.98	102.4 ± 55.1	125.1 ± 34.6	86.00 ± 28.16	95.70 ± 70.17	114.3 ± 57.3

a significant difference ($P<0.05$) from APR extract

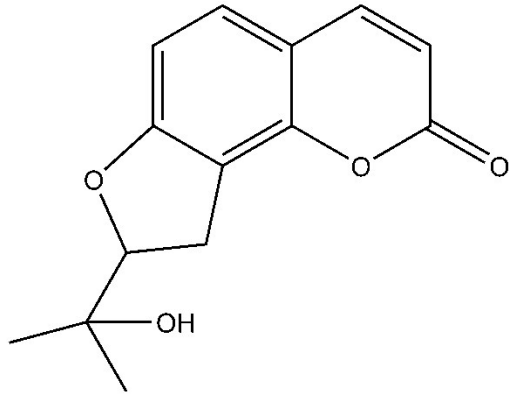
b extremely significant difference ($P<0.01$) from APR extract

c significant difference ($P<0.05$) from APR extract and columbianetin acetate (APR + 5mg)

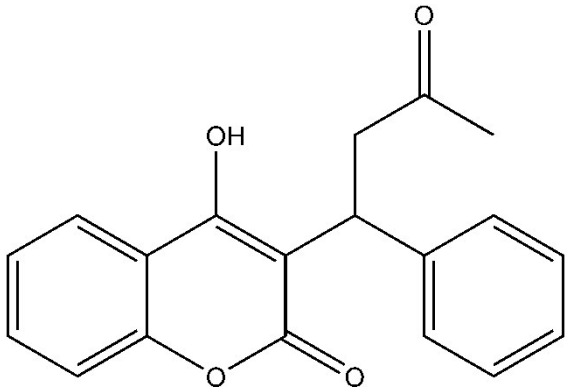
d extremely significant difference ($P<0.01$) from APR extract and columbianetin acetate (APR + 5mg)



Columbianetin acetate



Columbianetin



Warfarin (IS)

Fig.1 Chemical structures of columbianetin, columbianetin acetate and warfarin (IS).

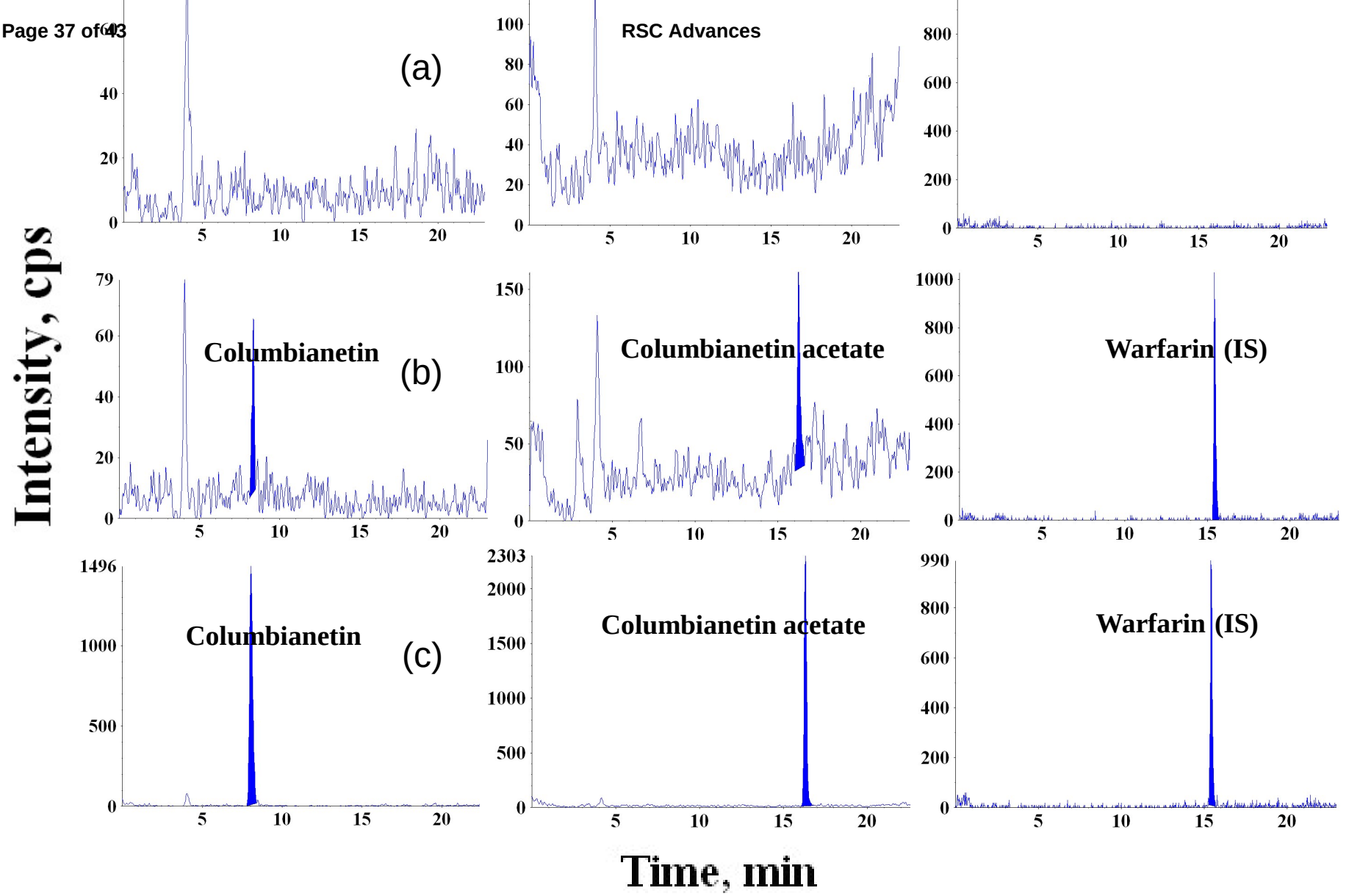


Fig 2. Representative chromatogram of (A) blank rat plasma,(B) blank rat plasma spiked with standard compounds at LLOQ and (C) real sample after administration of columbianetin acetate

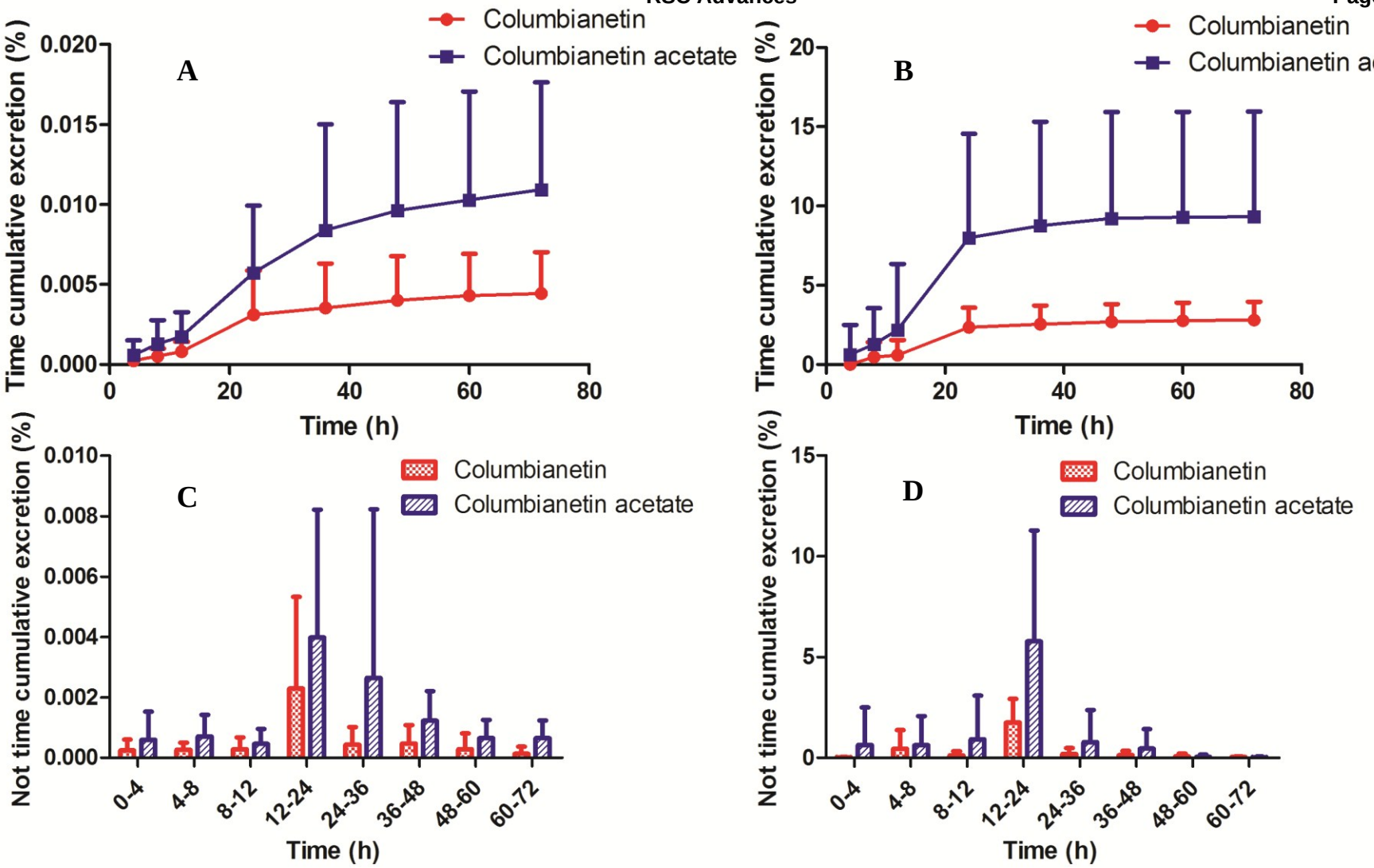


Fig.3 Time cumulative excretion percentage of columbianetin acetate and its metabolite columbianetin (A) in urine and (B) in feces; not time cumulative excretion percentage of columbianetin acetate and its metabolite columbianetin (C) in urine and (D) in feces.

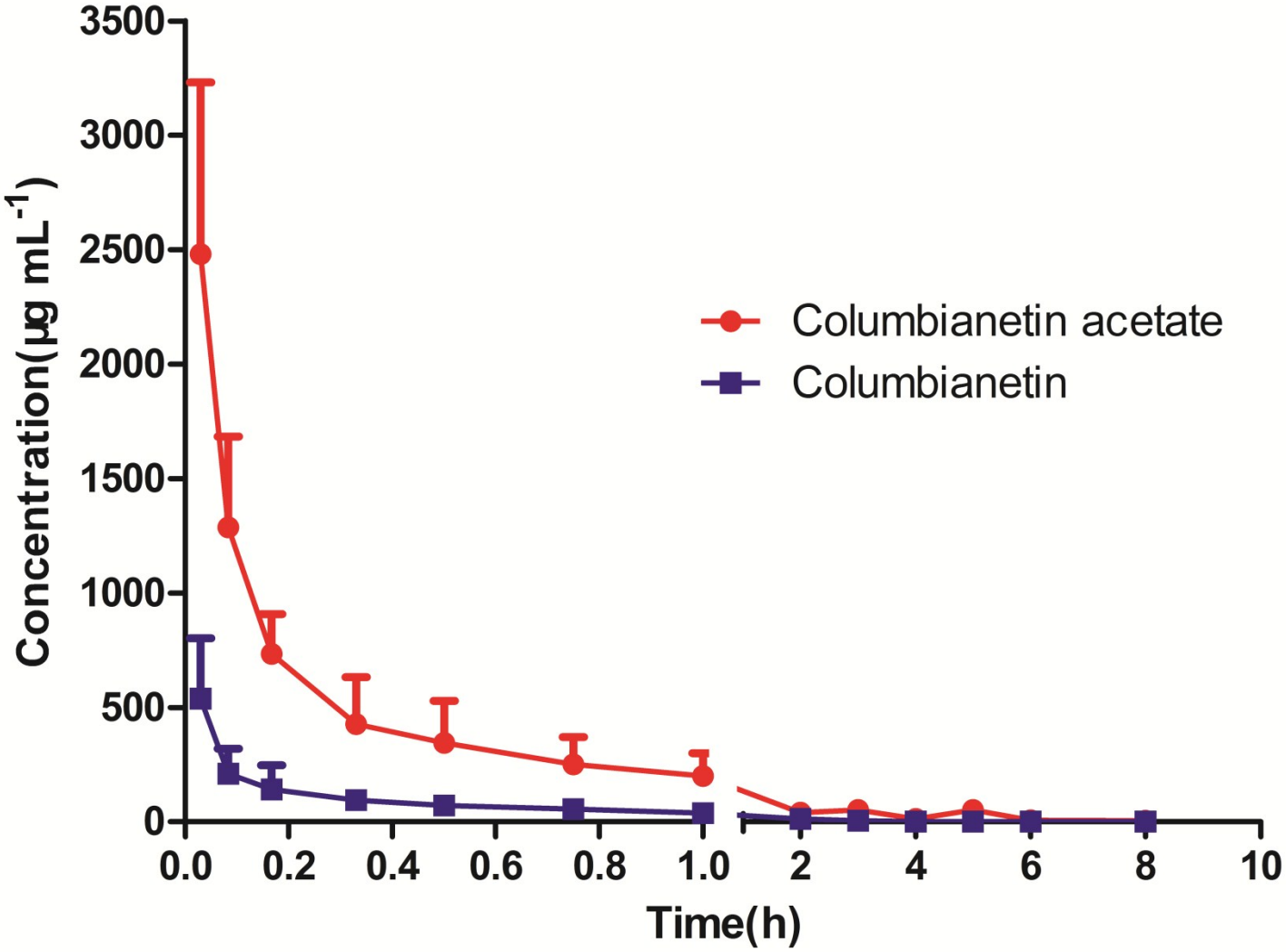


Fig.4 The mean plasma concentration-time profiles of columbianetin acetate and columbianetin after intravenous administration of pure columbianetin acetate.

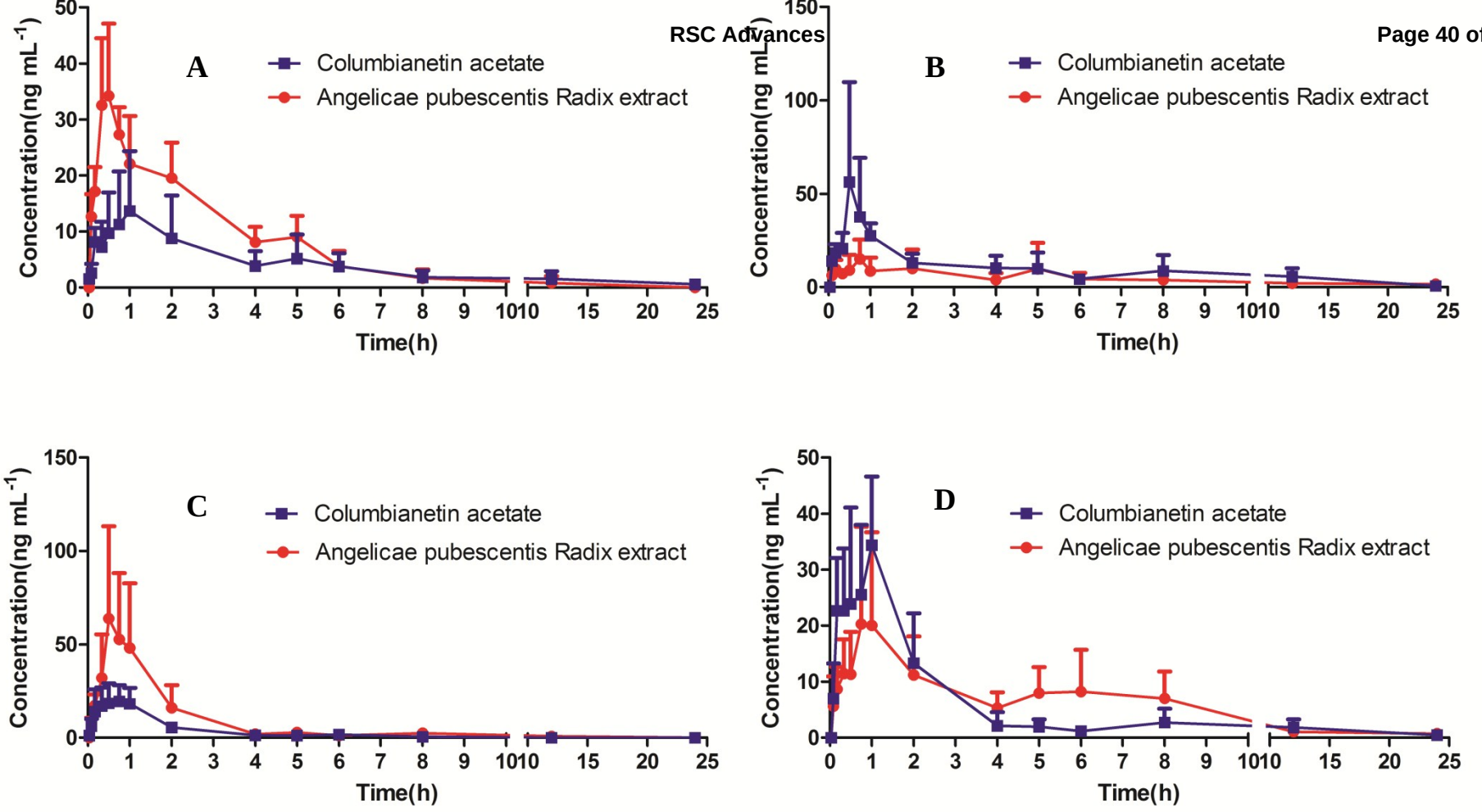


Fig.5 The mean plasma concentration-time profiles of (A) columbianetin acetate of i.g. 5 mg kg^{-1} columbianetin acetate and 5 mg kg^{-1} columbianetin acetate in extract (B) columbianetin acetate of i.g. 10 columbianetin acetate and 10 mg kg^{-1} columbianetin acetate in extract ; (C) columbianetin of of i.g. 5 mg kg^{-1} columbianetin acetate and 5 mg kg^{-1} columbianetin acetate in extract (D) columbianetin of of i.g. 10 mg kg^{-1} columbianetin acetate and 10 mg kg^{-1} columbianetin acetate in extract .

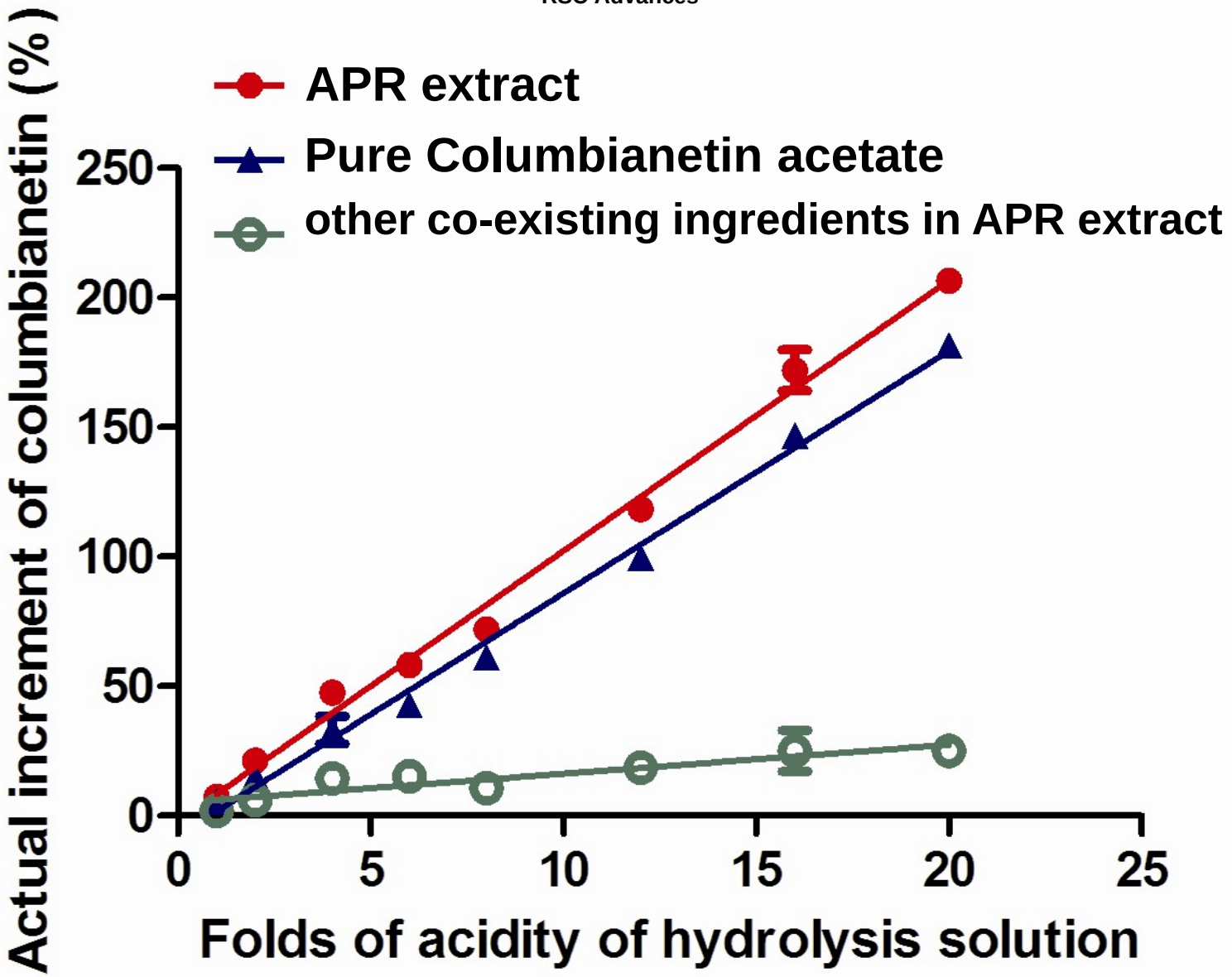


Fig.6 Relationship between percentage of actual increment of columbianetin and various folds of acidity of hydrolysis solution

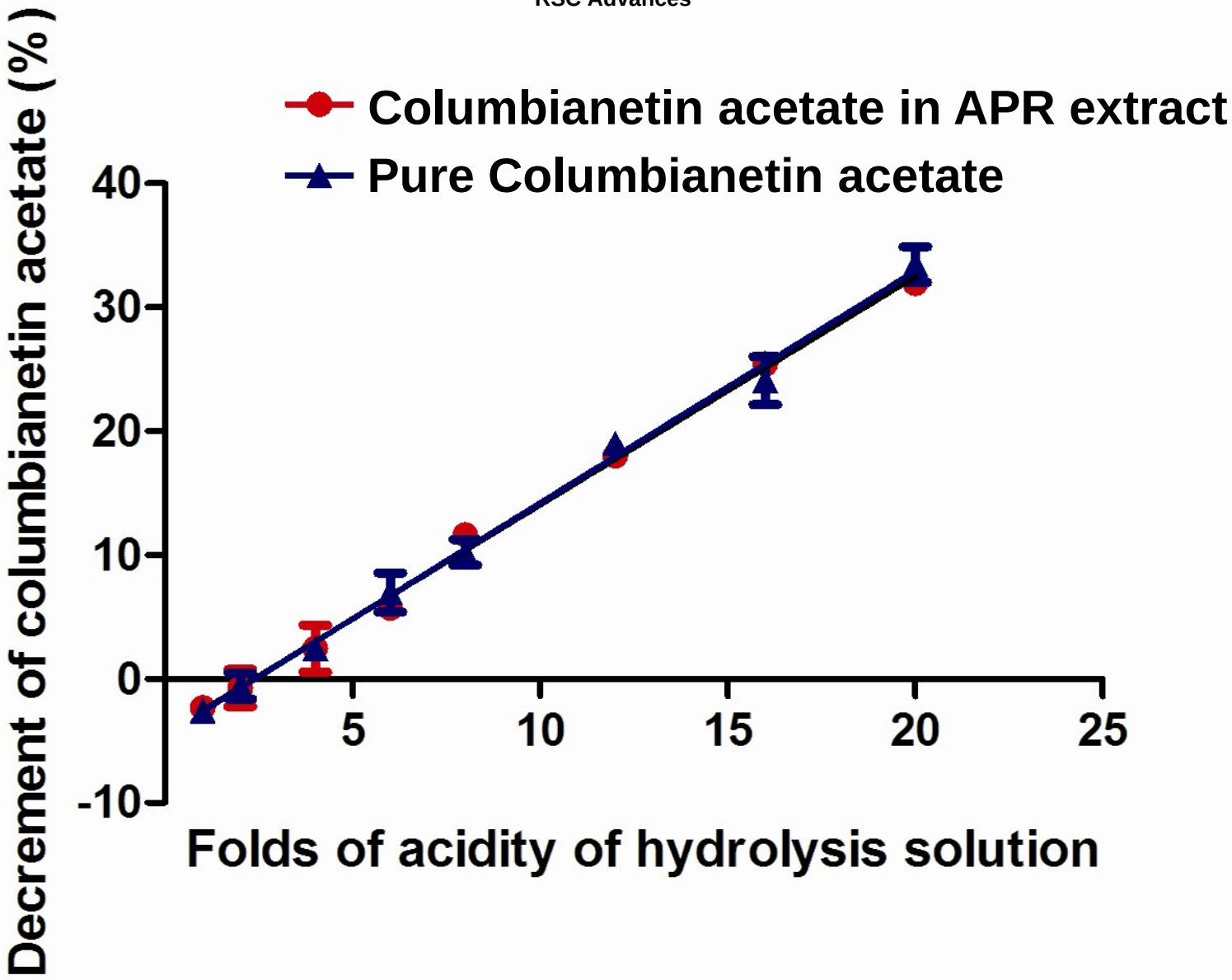
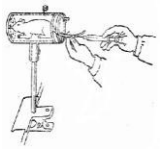


Fig.7 Relationship between percentage of decrement of columbianetin acetate and various folds of acidity of hydrolysis solution

***Angelicae pubescentis* Radix**

Columbianetin acetate

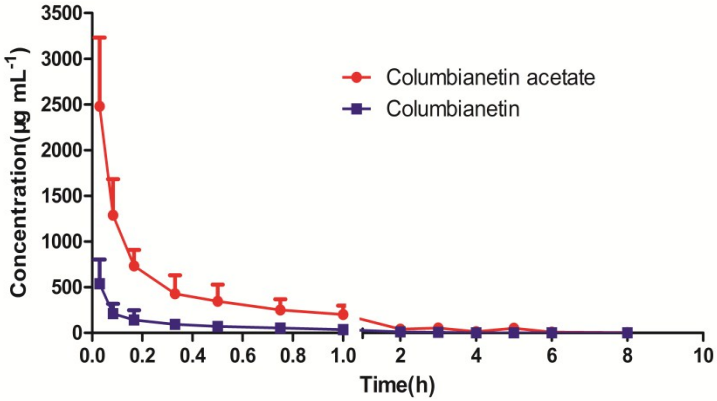
Extracts

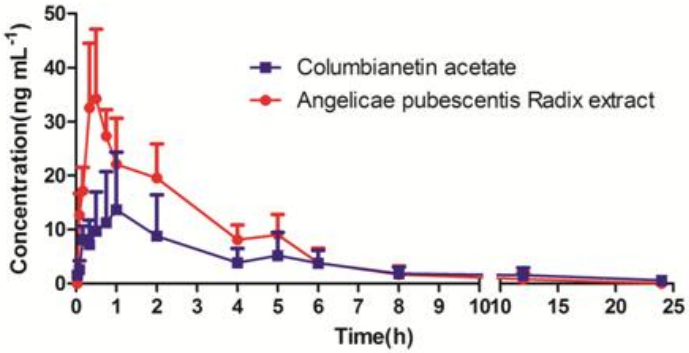


i.v.

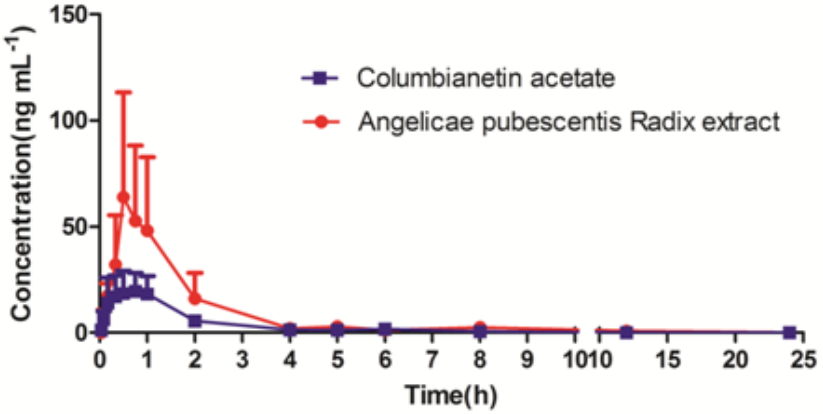
i.g.

i.g.





columbianetin acetate



columbianetin