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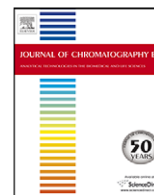

Determination of asiatic acid in beagle dog plasma after oral administration of *Centella asiatica* extract by precolumn derivatization RP-HPLC

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ABSTRACT

A novel precolumn derivatization reversed-phase high-performance liquid chromatography (RP-HPLC) method with UV–vis detection for the quantitative determination of total concentration of asiatic acid (AA) in beagle dog plasma is described. AA was extracted with n-hexane-dichloromethane-2-propanol (20:10:1, v/v/v) from plasma, which had been hydrolyzed by acid and derivatized with p-Toluidine. Chromatographic separation was achieved on a C_{18} column using gradient elution in a water–methanol system. Detection was set at UV wavelength of 248 nm. A calibration curve ranging from 0.01 to 1.5 μg/mL was shown to be linear, and the lower limit of quantification (LLOQ) was 0.01 μg/mL. The intra- and inter-day precisions which were determined by three different concentrations (0.05, 0.2 and 0.8 μg/mL) ranged from 4.4% to 13.1% and 4.6% to 14.2%, respectively. Mean extraction recoveries were no less than 65% for AA and ursolic acid (IS). Plasma samples containing asiatic acid were stable for 30 days at −20 °C. The method was successfully applied to a pharmacokinetic study in beagle dogs after oral administration of *Centella asiatica* extract, and the main pharmacokinetic parameters obtained were: $T_{1/2}$, 4.29 h; T_{max}, 2.70 h; C_{max}, 0.74 μg/mL; AUC_{0-t} and $AUC_{0-\infty}$, 3.74 and 3.82 μg h/mL, respectively.

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1. Introduction

Centella asiatica is a plant belonging to the *Umbelliferae* family, hydrocotyle order, which has been used for many years in the treatment of venous ulcers and venous hypertension for its activity on connective tissue metabolism and endothelial integrity. Asiaticoside, one of the principle terpenoids in *C. asiatica*, is presumed to be converted to asiatic acid (AA) (Fig. 1A) by the caecal microflora and the metabolic product is responsible for the therapeutic effects [1–3].

In the past decades, many analytical methods have been developed for the determination of AA. It is known that thin-layer chromatography and mass spectrometry could be used for the identification of *C. asiatica* extracts [4]. For quantitative determination of AA, gradient elution HPLC method with UV–vis detection at low wavelength (200–206 nm) is often employed [5–7]. These methods are usually applied in plant materials. For biological samples, analytical methods such as isotopic tracer method [8], gas chromatography–mass spectrometry (GC–MS) method [3,9] and HPLC method with UV–vis detection at 200 nm [2], have been established for the determination of AA and its derivative (AS2-006A, ethoxymethyl 2-oxo-3, 23-O-isopropylideneasiatate) [10]. However, the above-mentioned HPLC method [2] has not full validated. Besides, AA has absorption at low UV wavelength range. But if UV wavelength is set at 200 nm, interferences from endogenous substances may be significant and may affect the specificity of HPLC. In this paper, a novel precolumn derivatization HPLC method is present, with UV detection at 248 nm for the investigation of total concentration of AA in beagle dogs plasma after oral administration of *C. asiatica* extract. p-Toluidine (PTD) as the derivatizing reagent can be coupled with the free carboxylic acid group of AA. After derivatization, the maximum absorption wavelength of the derivative was 248 nm at which interferences became insignificant and the response was greatly enhanced compared with unmodified AA detected at 200 nm. Moreover, a homologue of ursolic acid (UA) (Fig. 1B) was used as an internal standard (IS). Analysis was conducted on a C_{18} column using gradient elution in a water–methanol system. In summary, a sensitive and selective HPLC method with UV–vis detection was established and successfully applied to a pharmacokinetics study in beagle dogs after oral administration of *C. asiatica* extract.

2. Experiment

2.1. Chemicals and reagents

AA (purity > 98%, HPLC) and *C. asiatica* extract (composed mainly of 72% asiaticoside and 6% madecassoside, HPLC; the water

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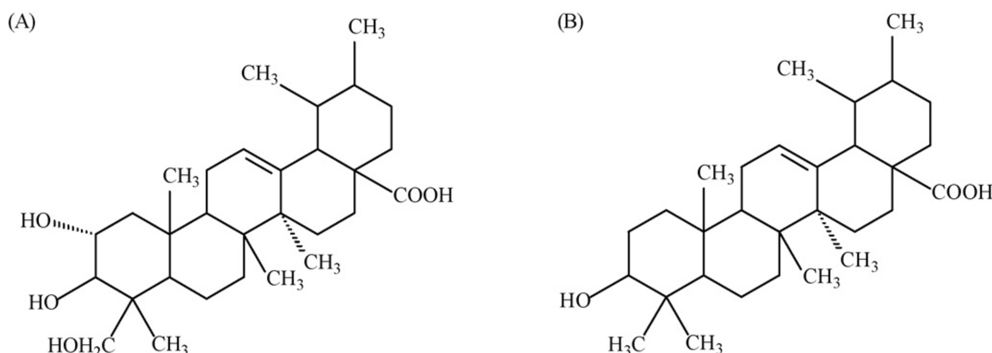

Fig. 1. Structures of asiatic acid (A) and ursolic acid (B).

soluble extract obtained from the aerial parts of *C. asiatica* (L.) Urban) were kindly donated by Guangxi Institute for Food and Drug Control (Guangxi, China). *C. asiatica* extract capsules were prepared in laboratory (each capsule contains about 190 mg asiaticoside). UA (IS) (purity > 98%, HPLC) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). PTD (purity > 99%, HPLC) was obtained from Hengxin Chemical Reagent Company (Shanghai, China). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC·HCl) was purchased from Sigma Chemical Company (St. Louis, MO, USA). Methanol was of HPLC-grade, and purchased from Merck Company (Darmstadt, Germany). n-Hexane, dichloromethane and 2-propanol were of analytical grade, and purchased from Hangzhou Chemical Reagent Company (Hangzhou, China). n-Hexane, dichloromethane, 2-propanol and water were doubly distilled. All solutions were filtered through a 0.45 μm pore-size membrane filter before use.

2.2. HPLC system

The HPLC system consisted of two LC-10ATvp pumps, a manual injector with 50 μL fixed loop and a SPD-10Avp UV–vis detector (Shimadzu Corporation, Kyoto, Japan). After precolumn derivatization, AA and UA derivatives were separated on a Diamonsil™ C_{18} column (250 mm × 4.6 mm, i.d., 5 μm, Dikma, USA) with a C_{18} guard column (8 mm × 4.6 mm, i.d., 5 μm, Dikma, USA). The mobile phase solvent A was HPLC-grade water and the solvent B was methanol. A gradient elution was used to elute AA and UA derivatives from the column (0–8 min, 15% A, 85% B; 8–36 min, 15% A, 85% B → 8% A, 92% B; 36–37 min, 8% A, 92% B → 0% A, 100% B; 37–43 min, 0% A, 100% B). The flow rate was set at 1.0 mL/min and the temperature was maintained at 25 °C. Detection was set at UV wavelength of 248 nm. The injected volume was 50 μL. The Chromatographic data were recorded and processed using a N2000 Version 3.0 (Zhejiang University, Hangzhou, China).

2.3. Preparation of solutions

The stock solutions of AA (100 μg/mL) and UA (10 μg/mL) were prepared in methanol and stored at 4 °C before use. The solutions stored at 4 °C were found to be stable for at least 3 months. AA stock solution was diluted to 0.2, 1, 8 and 30 μg/mL with methanol, and appropriate volumes of these AA solutions and 40 μL of UA solution were taken into tubes, respectively. The tubes were dried by a gentle stream of nitrogen, then the residues were reconstituted with 1 mL dog plasma to yield final respective concentrations of 0.01, 0.05, 0.1, 0.2, 0.4, 0.8 and 1.5 μg/mL. Quality control (QC) samples (0.05, 0.2 and 0.8 μg/mL) were prepared in the similar way.

2.4. Sample preparation

To 1.0 mL of dog plasma, 40 μL of IS solution was added. The mixed plasma sample was hydrolyzed by 250 μL hydrochloric acid (10 mol/L) in a water bath at 85 °C for 2 h and then extracted with 6 mL n-hexane-dichloromethane-2-propanol (20:10:1, v/v/v). After vortexing for 10 min, sample was centrifuged at 5500 rpm for 10 min. The organic layer was transferred to another tube and evaporated to dryness in a water bath at 35 °C under a stream of nitrogen.

To the residue, 150 μL of PTD solution (2.5 mg/mL, prepared freshly in methylene dichloride) and 350 μL of EDC solution (2.5 mg/mL, prepared freshly in methylene dichloride) were added. The mixture was vortexed gently for 5 min and then kept at 30 °C for 3 h. The solution was evaporated to dryness under nitrogen. The residue was reconstituted with 200 μL of methanol–water (85:15, v/v) and centrifuged at 12,000 rpm for 5 min. An aliquot of 50 μL of the resulting solution was injected into the HPLC system for analysis.

2.5. Pharmacokinetics study

2.5.1. Pharmacokinetics study in beagle dogs after oral administration of Centella asiatica extract

Five beagle dogs (male, 12 ± 1.5 kg) were purchased from Jia-an Laboratory Animal Company (Jiaxing, China). Diet was prohibited for 12 h before the experiment while water was taken freely. Each dog received two capsules of *C. asiatica* extract (containing 540 mg total glucosides, in which asiaticoside accounts for about 72%) by oral administration. Blood samples (about 2 mL) were collected into heparinized tubes through limb veins at 1 day before oral administration and at 0.5, 1.5, 2.5, 3.5, 4.5, 5.5, 6.5, 8, 10, 12, 18, and 24 h post-oral administration. Samples were centrifuged at 5500 rpm for 10 min to separate plasma, which was stored at −20 °C before analysis. The plasma samples were thawed at room temperature just before assaying. This animal study was approved by the Zhejiang Academy of Medical Sciences Institutional Animal Care and Use Committee.

2.5.2. Pharmacokinetic analysis [11]

The pharmacokinetic parameters were calculated with the Drug and Statistics computer program (PKS, Shanghai Magnsoft Consulting). The maximum plasma concentration (C_{max}, observed data) and the time to reach maximum plasma concentration (T_{max}, observed data) were both obtained directly from the measured data. The area under the plasma concentration–time curve (AUC) and the area under the first-moment time curve (AUMC) were calculated by the trapezoidal method, and extrapolated to infinity using the last measurable plasma concentration and the terminal elimination

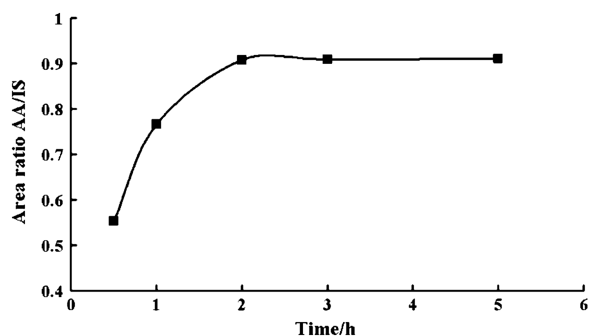

Fig. 2. Effect of hydrolysis time on the formation of AA derivative.

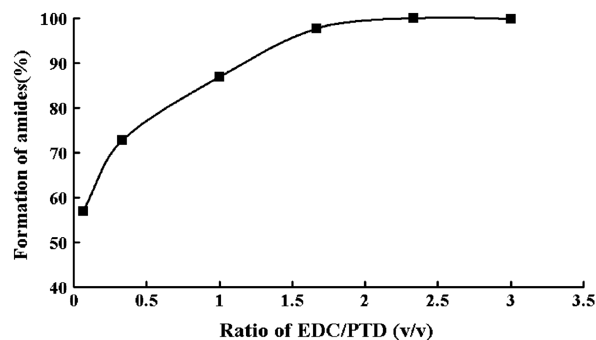

Fig. 4. Effect of amounts of derivatizing agents on the derivatization.

rate constant. Mean residence time (MRT) was calculated using the equation MRT = AUMC/AUC. The terminal elimination half-life ($t_{1/2}$) was derived by linear regression analysis of the terminal phase of the plasma concentration–time curve.

3. Results and discussion

3.1. Hydrolysis

It is reported that most AA can be metabolized to asiatic acid glucuronide and asiatic acid sulphate in vivo [4]. The drug in plasma is mainly present as the asiatic acid glucuronide, asiatic acid sulphate and a small percentage of free AA. Therefore the investigation of total concentration of AA in plasma would be suitable to illuminate the pharmacokinetics of *C. asiatica* extract (asiaticoside is the principal constituent of the extract) after oral administration. As a result, in our study, plasma was hydrolyzed by hydrochloric acid before extraction. Asiaticoside was used as the model drug to investigate the effect of time on the hydrolyzing of plasma. Quantitative asiaticoside and IS solutions were added to 1.0 mL of blank dog plasma, then the mixed plasma samples were hydrolyzed by 250 μL hydrochloric acid (10 mol/L) in a water bath at 85 °C for 0.5, 1, 2, 3 and 5 h, respectively. The samples were processed as described in Section 2.4. As seen from Fig. 2, no significant increases of peak areas ratio of AA vs. the IS were observed when the plasma was hydrolyzed above 2 h.

3.2. Derivatization

Referring to similar method [11], the covalent attachment of PTD to AA was achieved by the formation of amide bond of primary amino group of PTD with carboxylic acid group of AA. After the covalent coupling of PTD, the UV absorption of AA and UA increased remarkably. The proposed derivatization scheme is shown in Fig. 3.

3.2.1. Effect of amounts of derivatizing agents on the derivatization

Samples added with different volumes of EDC and PTD were used to investigate the effect of amounts of coupling agents on the derivatization. Quantitative AA was taken into tubes and the samples were added with different volume of EDC (10, 50, 150, 250, 350, 450 μL, 2.5 mg/mL) and 150 μL PTD (2.5 mg/mL). As seen from Fig. 4, no significant increases of peak area of AA were observed when the volume ratio of EDC vs. PTD (v/v) was above 7:3.

3.2.2. Effect of reaction time on the derivatization

According to the result of Section 3.2.1, the samples were added with 350 μL EDC (2.5 mg/mL) and 150 μL PTD (2.5 mg/mL). Derivatization were carried out at 30 °C for 0.5, 1, 2, 3, 5 and 7 h, respectively. As seen from Fig. 5, after 3 h, no significant increases of peak area of AA were observed, so the reaction time was set at 3 h in the following plasma sample analysis.

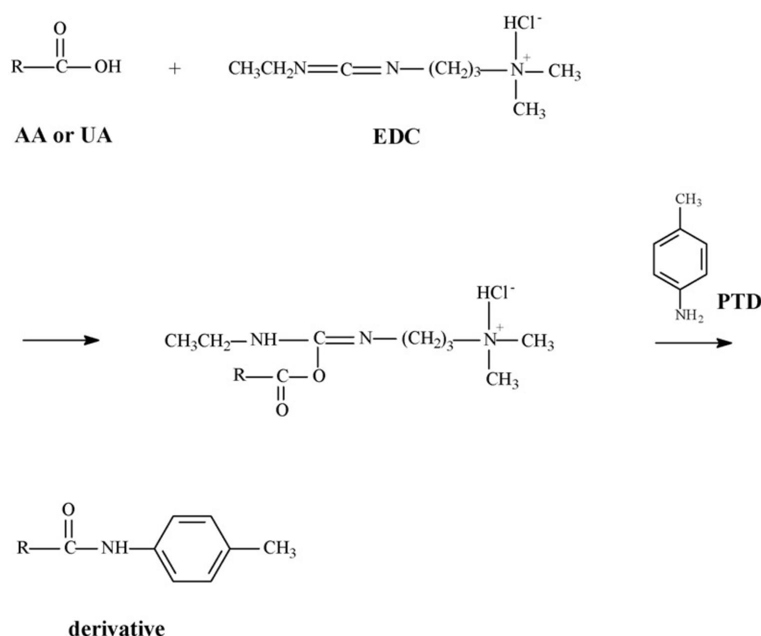

Fig. 3. Derivatization scheme of AA (UA) with PTD.

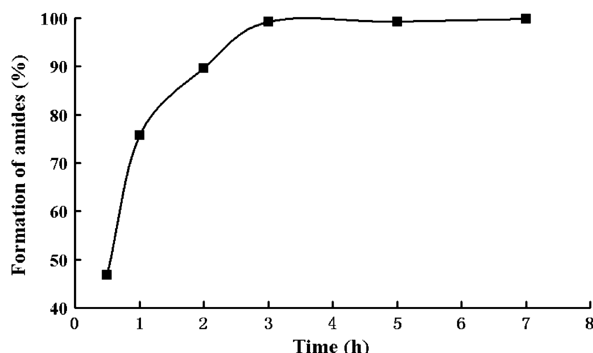

Fig. 5. Effect of reaction time on the derivatization.

3.3. Method validation

3.3.1. Specificity

AA and UA are pentacyclic triterpenes and their structures are very similar. However, in the isocraic elution (85% methanol), the retention time of AA and UA derivatives are very different (about 12.5 and 50 min, respectively). Thus, gradient elution procedure was employed. After gradient elution, the retention time of UA derivative shift to an earlier time while that of AA derivative only changed slightly. Moreover, suitable adjustment of gradient elution procedure could enhance specificity of HPLC.

Representative chromatograms of blank plasma, plasma spiked with AA and UA, and a dog plasma sample after administration of *C. asiatica* extract are presented in Fig. 6. Five blank plasma samples were prepared. No endogenous peaks from plasma were found to interfere with the elution of AA and UA. This HPLC system is specific for determination of AA in beagle dog plasma, and its matrix effects as well as ion suppression of the plasma samples were not significant.

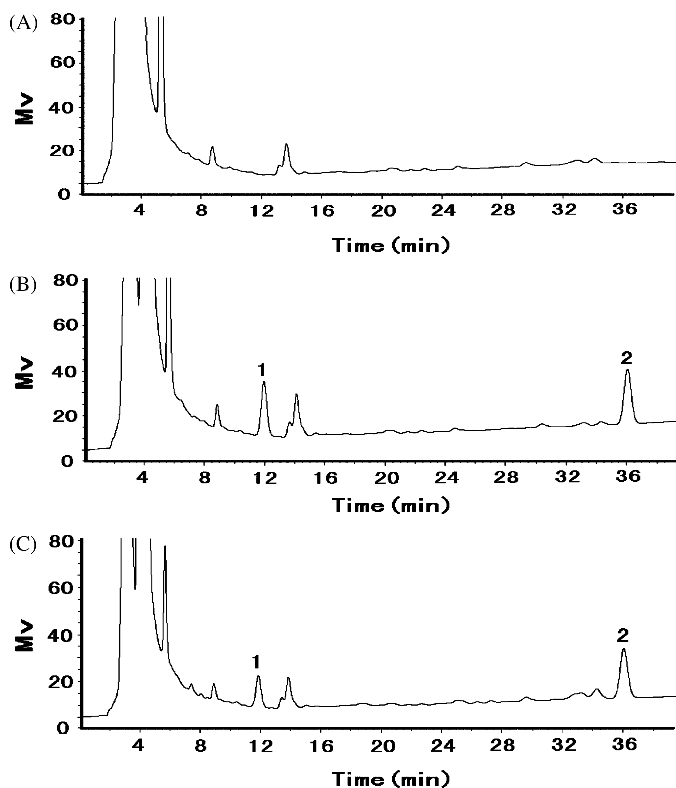

Fig. 6. Representative HPLC chromatograms of blank dog plasma(A); sample of blank plasma spiked with AA (0.4 μg/mL) and IS of UA (0.4 μg/mL) (B); dog plasma sample after oral administration of *Centella asiatica* extract at 5.5 h spiked with IS of UA (C). 1: asiatic acid derivative. 2: IS, ursolic acid derivative.

Table 1
Precision, accuracy and recovery for assay of AA in spiked plasma (mean ± SD, $n = 5$).

Conc. spiked (μg/mL)	Extraction recovery (%)	Accuracy (%)	Relative standard deviation (%)	
			Intra-day	Inter-day
0.05	65.2 ± 14.2	105.1 ± 13.8	13.1	14.2
0.2	69.4 ± 5.1	101.9 ± 5.9	5.8	6.2
0.8	68.7 ± 4.1	101.6 ± 4.5	4.4	4.6

3.3.2. Linearity and the limit of quantification

The calibration curve for the sample assay described in Section 2.4 was found to be linear over the concentration range 0.01–1.5 μg/mL for AA. Peak area ratios (y) of AA vs. the IS were measured and plotted against the concentration (x) of AA. The regression equation of the calibration curve was $y = 6.4361x + 0.221$ ($r = 0.9996$). The lowest concentration of the calibration curve, also represented as the lower limit of quantification (LLOQ, S/N > 10), was 0.01 μg/mL with RSD of 14.6% ($n = 5$). The limit of detection (LOD) was 2.5 ng/mL (S/N = 3).

3.3.3. Precision and accuracy

The intra- and inter-day precision and accuracy were obtained by analyzing the spiked samples at concentrations of 0.05, 0.2, and 0.8 μg/mL. For intra-day precision and accuracy, five replicate QC samples at each concentration were assayed on the same day. The inter-day precision and accuracy were evaluated on five consecutive days. The results were summarized in Table 1. The intra-day and inter-day precisions were within 15%. The recovery of the assay was between 101.6–105.1%.

3.3.4. Extraction recovery

Extraction efficiency of AA from the spiked samples were evaluated at concentrations of 0.05, 0.2, and 0.8 μg/mL ($n = 5$). The plasma samples containing a known amount of AA were prepared as described in Section 2.4. In another set of tubes, equivalent amounts of AA in pure solvent were added and followed by the derivatization procedure. Recovery was assessed by comparing the peak areas of AA derivative obtained from the extracted plasma with those obtained from equivalent amounts of standard with the same concentration. The recoveries of AA were summarized in Table 1. The average of this analytical method varied from 65.2% to 69.4% (Table 1). The extraction recovery of IS with concentration of 0.4 μg/mL was 67.0 ± 6.2%.

Extraction recovery seemed relatively low (<70%) in the present study, for the drug of AA is slightly soluble in many organic solvents (such as dichloromethane, trichlormethane and ethyl acetate), while freely soluble in acetonitrile or alcohols (such as methanol and 2-propanol). We had tried various single solvent and their mixtures to extract AA from plasma, but extraction recovery was unsatisfactory. Finally, a mixture of hexane-dichloromethane-2-propanol (20:10:1, v/v/v) was found to be optimal for producing a clean blank plasma chromatogram and yielding the high recovery for AA and IS.

3.3.5. Stability

The stability of AA in dogs plasma during storage at −20 °C was studied at concentrations of 0.05, 0.2, and 0.8 μg/mL. The results demonstrated that there were no notable differences among the observed concentrations on days 7, 15 and 30. Therefore it can be proved that AA was stable in beagle dogs plasma for at least 30 days when stored at −20 °C. The RSD were 14.8%, 7.2%, 5.5% at concentrations of 0.05, 0.2 and 0.8 μg/mL, respectively.

Table 2
Robustness of the method for QC samples (0.2 μg/mL) ($n = 3$).

Parameters	Modification	Retention time (min)	Mean concentration found (μg/mL)	Difference from normal conditions (%)
pH of mobile phase	3.0	12.44	0.197	−3.0
	4.0	12.38	0.198	−2.5
	5.0	12.12	0.206	+1.5
	Water[a]	11.96	0.203	
Column temperature (°C)	20	12.30	0.199	−2.0
	25[a]	11.96	0.203	
	30	11.78	0.208	+2.5

[a] Normal conditions.

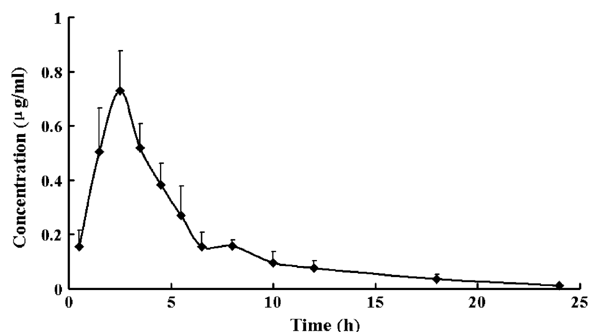

Fig. 7. Plasma concentration–time curve of AA in dogs after oral administration of *C. asiatica* extract ($n = 5$). Vertical bars represent SD.

3.3.6. Robustness

The robustness of the method was performed by evaluating small variations in column temperature (20, 25 and 30 °C) and in pH of mobile phase (pH 3.0, 4.0, 5.0 phosphate buffer and neutral HPLC-grade water (Fisher Scientific, Pittsburgh, USA) as the water phase). By increasing the column temperature and the pH of mobile phase resulted in a decrease of the retention time of AA derivative. The method had good robustness, for the concentration of QC samples was not significantly affected by these small changes (Table 2). Moreover, minor change of gradient elution procedure (shifts of ±1% of the percentage of mobile phase methanol at 36 min) had no significant impact on the specificity, resolution and peak shape of AA and UA derivatives.

3.4. Pharmacokinetics study

The developed and validated HPLC method described herein was applied to determine total concentration of AA in beagle dogs plasma after oral administration of *C. asiatica* extract capsule. Main pharmacokinetic parameters obtained were: $T_{1/2}$, 4.29 ± 0.70 h; T_{max}, 2.70 ± 0.45 h; C_{max}, 0.74 ± 0.13 μg/mL; AUC_{0-t} and $AUC_{0-\infty}$, 3.74 ± 0.42 and 3.82 ± 0.44 μg h/mL; MRT_{0-t} and $MRT_{0-\infty}$, 5.72 ± 0.58 and 6.20 ± 0.72 h, respectively. The mean plasma concentration–time curves profile is shown in Fig. 7. The pharmacokinetic results suggest that the plasma AA concentration rise quickly to the maximum concentration (C_{max}) of 0.74 μg/mL at 2.7 h (T_{max}) after oral administration of *C. asiatica* extract. Comparing the PK profiles in beagle dogs obtained with those in human [2,3], we found that the drug in beagle dogs had shorter T_{max} and longer $T_{1/2}$ which may result from different species. From the plasma concentration–time profile, we can see that AA had a second maximum concentration at 8 h, which was probably caused by the entero-hepatic circulation of drug [8]. The low total concentration of AA in beagle dogs plasma implies that drug is poorly absorbed by the intestine.

4. Conclusion

In conclusion, a simple, sensitive and selective precolumn derivatization HPLC method with UV detection for the determination of total concentration of AA in beagle dogs plasma was developed. The assay had been successfully applied to study the pharmacokinetics in beagle dogs after oral administration of *C. asiatica* extract.

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