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Determination of aesculin in rat plasma by high performance liquid chromatography method and its application to pharmacokinetics studies

QiuHong Chen [a], ShiXiang Hou [a,*], Jia Zheng [b], YueQi Bi [a], YuanBo Li [a], XiaoJiao Yang [a], Zheng Cai [a], XiangRong Song [a]

[a] *No. 17, Renmin Nan Road, West China School of Pharmacy, Sichuan University, Chendu 610041, PR China*
[b] *No. 30, Gaotanyan Street, Department of Medical Education, Third Military University, Chongqing 400038, PR China*

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Abstract

A sensitive and reproducible high performance liquid chromatography method with UV detection was described for the determination of aesculin in rat plasma. After deproteinization by methanol using metronidazole as internal standard (I.S.), solutes were evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was reconstituted in 100 μl of mobile phase and a volume of 20 μl was injected into the HPLC for analysis. Solutes were separated on a Diamonsil C_{18} column (250 mm × 4.6 mm i.d., 5 μm particle size, Dikma) protected by a ODS guard column (10 mm × 4.0 mm i.d., 5 μm particle size), using acetonitrile–0.1% triethylamine solution (adjusted to pH 3.0 using phosphoric acid) (10:90, v/v) as mobile phase (flow-rate 1.0 ml/min), and wavelength of the UV detector was set at 338 nm. No interference from any endogenous substances was observed during the elution of aesculin and internal standard (I.S., metronidazole). The retention times for I.S and aesculin were 10.4 and 12.4 min, respectively. The limit of quantification was evaluated to be 57.4 ng/ml and the limit of detection was 24.0 ng/ml. The method was used in the study of pharmacokinetics of aesculin after intraperitoneal injection (i.p.) administration in rats.

Keywords: Aesulin; Reversed-phase HPLC; Pharmacokinetics; Metronidazole; Plasma

1. Introduction

In recent years there has been a growing interest in *Cortex Fraxini* (Chinese name Qin-pi), which comes from the dry tegument of different categories of *Fraxini chinensis* Roxb and has been widely used in China for over 2000 years [1]. Recently, considerable attention has been focused on aesculin (AL)—the main effective constituent of *Cortex Fraxini*. AL have multiple biological functions including inhibition of xanthine oxidase, anti-oxidant activity, antitumor activity, and inhibitory effect on the growth of human breast cancer cells [2,3]. It also has the therapeutic effect of clearing away pathogenic heat, cure bacterial dysentery and trachitis nourishing the liver, diuresis, central inhibition, anti-anaphylaxis and improving vision [4–6]. In addition, it is effective in the treatment of diarrhoea and cough [6,7]. Furthermore, data indicate that AL has some inhibition effect on human nasopharyngeal carcinoma cell strain KB [8].

Many methods have developed to assay AL *in vitro* including high performance liquid chromatography (HPLC) [9], high performance capillary electrophoresis (HPCE) [1]. Capillary electrophoresis end-column amperometric detection [1,5,6,10], capillary zone electrophoresis [1], and non-aqueous capillary electrophoresis with UV detection [11]. However, the determination of AL in biological fluids was scarcely studied, except fluorospectrophotometry method, which was reported by Wang to assay AL in aqueous humor [12]. The shortcomings of fluorospectrophotometry are noticeable, and there is distinct interference when it is applied to assay AL in plasma. AL is a kind of glycoside, which is easy to be hydrolyzed *in vitro* to produce aesculetin, which is another effective component of *Cortex Fraxini*. A separation technology is prerequisite to detect AL in

Disclaimer: All experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals in Sichuan University, Chengdu, China, and conformed to the National Institutes of Health Guide for Care and Use of Laboratory Animals.

* Corresponding author. Tel.: +86 28 85501376; fax: +86 28 85502809.
E-mail address: cqhhong617@163.com (Q. H. Chen).

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plasma. And it is preferable to establish a simple and effective method for the quantitative analysis of AL in plasma or other biological fluids.

In this paper, a novel HPLC–UV method with metronidazole as I.S. is developed to assay AL in rat plasma specimens. This method was fully validated for its specificity, accuracy, precision and sensitivity. The LOQ and LOD of the assay are in the low ng/ml range. Good separation, high sensitivity and simple approach, without compromising the specificity, are the main advantages of such a technique. This method is also efficient in analyzing large numbers of plasma samples obtained form pharmacokinetics study after 10 mg/kg doses of AL to rat [7].

2. Materials and methods

2.1. Materials and reagents

AL ($C_{15}H_{16}O_9$; MW = 340.28) and metronidazole (I.S., $C_6H_9N_3O_3$; MW = 171.16) were obtained from National Institution for the control of pharmaceutical and biological products. The molecular structure of AL and metronidazole are illustrated in Fig. 1. Methanol (Hanbon, Jiangsu, China) and acetonitrile (Fisher) were HPLC grade. Orthophosphoric acid (H_3PO_4) and Triethylamine were analytical grade and purchased from Chengdu Fangzhou Sci. and Tech. Co., Ltd. All chemical and solvents were of analytical or HPLC grade. All standard solutions and mobile phases were prepared using glass-double distilled water and passed through a 0.22 μm membrane filter before use.

2.2. Preparation of calibration standard and quality controls (QC) samples

Stock solutions of AL and I.S were prepared by dissolving accurately weighed AL and metronidazole in methanol to yield final concentrations of 61.24 and 50 μg/ml, respectively. The stock solutions were stored at 4 °C (stable for at least 2 months) and brought to room temperature before use [13]. The standard solutions were prepared before use by diluting the AL stock solution with appropriate methanol: water (5:95) to final concentrations ranging from 0.57 to 30.62 μg/ml. The internal standard solution was prepared by diluting the I.S. stock solution with water to yield a concentration of 5.0 μg/ml. Sprague–Dawley rats (180–220 g) were obtained from the Experimental Animal Center of Sichuan University. After anesthetized with ether via nasal, rat was decapitated and the blood was rapidly collected in heparinized polythene tubes. The blank plasma was separated by immediate centrifuging at 3000 rpm for 10 min and stored at −20 °C until required. The standard solution was diluted with blank rat plasma to yield the calibration standard solutions of 57.4, 95.7, 191.4, 382.8, 765.5, 1531.0 and 3062.0 ng/ml. Quality control (QC) samples were prepared to the target concentrations of approximately 57.4, 67.0, 114.8, 765.5 and 2449.6 ng/ml in the same way as the plasma samples for calibration. The QC samples were then divided into100 μl aliquots in tightly closed microtubes and kept frozen at −20 °C until required.

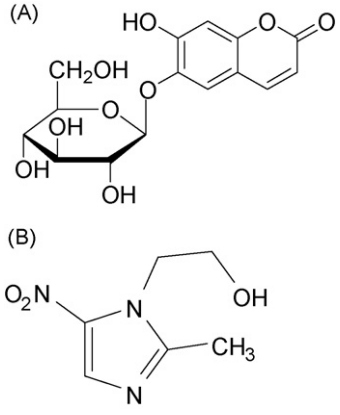

Fig. 1. Structure of aesculin (AL) (A) and metronidazole (B).

2.3. Sample preparation

To each of disposable plastic tubes with 100 μl plasma samples (calibration standards, QC samples, pharmacokinetics plasma samples), 10 μl of I.S. (metronidazole, 5 μg/ml) was added except the blank plasma sample. After vortexed for 5 min on a vortex mixer (Jiangxi Medical Instrument Group, China), 480 μl of methanol was added to each of these plasma samples. The obtained solution was vortexed for 10 min at room temperature. The mixture was centrifuged at 12,000 rpm for 20 min to remove any protein and the supernatant was removed into1.5-ml glass tubes and evaporation was completed to dryness under nitrogen at 40 °C. After adding 100 μl of the mobile phase, vortexing (5 min) and centrifuging (12,000 rpm for 20 min), a volume of 20 μl of the supernatant was injected into the HPLC for analysis.

2.4. High performance liquid chromatographic apparatus and chromatographic conditions

The HPLC system consisted of an Agilent chemistation Rev. A. 08.03 system (HP1100, Agilent, America) equipped with a G1311A Quaternary pump, a fixed injection-loop of 50 μl, and a G1314A variable wavelength detector operated at a wavelength of 338 nm. The analytical column employed was a Diamonsil C_{18} column (250 mm × 4.6 mm i.d., 5 μm particle size) (Dikma) and protected by a ODS guard column (10 mm × 4.0 mm i.d., 5 μm particle size). The mobile phase comprised of acetonitrile–0.1% triethylamine solution (adjusted to pH 3.0 using phosphoric acid) (10:90, v/v), was filtered through a 0.22 μm cellulose membrane filter (Auto Science, Tianjin, China). The mobile phase was then pumped through the system at a rate of 1.0 ml/min. Twenty microliters of sample solution was then injected onto the column. The chromatograph run required 15 min for completion. Separation was achieved at 30 °C. All chromatograms obtained were evaluated by the peak area ratio of AL to the I.S. The calibration curve was plotted with the peak area ratio of AL to the I.S against the plasma con-

centration of AL. The data demonstrated a linear function for AL according to the equation:

$$Y = aX + b$$

where Y is the plasma concentration of AL (ng/ml), X the peak area ratio of AL to the I.S, a the slope and b is the intercept.

2.5. Validation of the method

For method validation and linearity studies, blank plasma samples obtained from Sprague–Dawley rats (180–220 g) were used ($n = 6$). The specificity of the assay was evaluated by comparing between the rat blank plasma sample and rat plasma sample spiked with AL and the I.S. The efficiency of the extraction procedure was observed at concentrations of 114.8, 765.5 and 2449.6 ng/ml. Recovery was calculated by comparing the respective peak areas of the chromatograms of the extracted samples with the untreated standards solution containing an equivalent amount of the compounds. Calibration curves were constructed by linear least-squares regression analysis plotting of peak-area ratio (AL/I.S.) versus the drug concentrations. The lower limit of quantification (LOQ) was defined as the lowest concentration with relative standard deviation (R.S.D.) of less than 20% and accuracy of 80–120%. The limit of detection value (LOD) in rat plasma was calculated as the amount of the injected sample, which resulted in a signal-to-noise ratio of 3. The accuracy and precision of the method were evaluated with QC samples at concentrations of 57.4, 67.0, 114.8, 765.5 and 2449.6 ng/ml on three consecutive days, accompanied by a standard calibration curve on each analytical run.

The quality control (QC) samples were assayed under several different conditions to assess the stability of AL in rat plasma. One set of QC samples was stored at room temperature (approximately 22–25 °C) for 24 h in volumetric flask. The stability of the sample at room temperature was evaluated by comparing the assay results of the stored QC samples with that of the freshly thawed QC samples. Another set of QC samples was subjected to three freeze-thaw cycles and was then assayed to evaluated freeze-thaw stability of AL in rat plasma. Long-term stability was studied by assaying samples that had been stored at −20 °C for a certain period of time (0, 5, 10 and 30 days). AL was considered stable under storage conditions if the assay percent recovery was found to be 90–110% of the nominal initial concentration.

2.6. Application of the method

Sprague–Dawley male rats (180–220 g) were administered AL by i.p. injection at a dose of 10 mg/kg (∼2 ml) [7]. Then 0.3 ml blood samples *via* orbital veins were collected in heparinized 1.5 ml polythene tubes at 5, 10, 15, 30 min, 1, 1.5, 2, 3, 4, 6, 8 and10 h after injection, and the blood samples were immediately centrifuged at 3000 rpm for 10 min. A 100 μl volume of plasma was finally obtained and stored at −20 °C until required. The AL plasma concentration in the samples were determined using the above-mentioned HPLC method. The concentration data, at each time point, represented the mean ± standard deviation obtained from five rats. Pharmacokinetics parameters were calculated using DAS software.

3. Results and discussion

3.1. Specificity

No endogenous interference was found at the retention times of AL and the I.S. Representative chromatograms for blank rat plasma, AL standard and I.S. standard in methanol and rat plasma spiked with AL (765.5 ng/ml) and the I.S. (500.0 ng/ml) are shown in Fig. 2A–C, respectively. The I.S. and AL were well resolved with respective retention times of 10.4 and 12.4 min. Fig. 2D represents the chromatogram of plasma sample obtained at 1.5 h from SD rats after the i.p. treatment with 10 mg/kg of AL.

3.2. Linearity, limit of detection, precision and accuracy

The standard calibration curves were linear over the concentration ranges of 57.4–3062.0 ng/ml for AL with a correlation coefficient of 0.999. The linear regression equation for AL rat plasma concentration was: $Y = (595.27)X + (9.10)$ ($r^2 = 0.999$), where Y is the plasma concentration of AL and X is the peak area ratio of AL to the I.S. The limit of quantification (LOQ) in rat plasma for AL was 57.4 ng/ml. The limit of detection (LOD) in rat plasma for AL was 24.0 ng/ml, determined as the concentration with signal-to-noise ratio of 3.

Intra- and inter-day precision (as relative standard deviation (R.S.D.)) and accuracy were based on assay of the QC samples at concentrations of 57.4, 67.0, 114.8, 765.5, and 2449.6 ng/ml. The result of intra-day and inter-day precision and accuracy of the QC samples for AL in rat plasma are presented in Table 1.

3.3. Recovery

To determine the recovery of AL in rat plasma, an aliquot of blank rat plasma was spiked with AL to achieve a final concentration of 114.8, 765.5, and 2449.6 ng/ml. The plasma samples

Table 1
Assay precision and accuracy of AL in rat plasma

AL nominal concentration (ng/ml)	AL measured concentration (ng/ml)	R.S.D. (%)[a]	Accuracy (%)	n
Inter-day assay				
57.4	66.10 ± 6.74	10.2	115.2	6
67.0	70.62 ± 5.72	8.10	105.4	6
114.8	107.9 ± 6.73	6.23	94.0	6
765.5	726.0 ± 31.6	4.35	94.8	6
2449.6	2443.0 ± 52.0	2.13	99.8	6
Intra-day assay				
57.4	62.33 ± 7.73	12.4	108.6	6
67.0	66.17 ± 7.01	10.6	98.8	6
114.8	107.2 ± 11.4	10.6	93.4	6
765.5	697.7 ± 57.0	8.17	91.1	6
2449.6	2286.4 ± 145.8	6.38	93.3	6

[a] R.S.D., relative standard deviation.

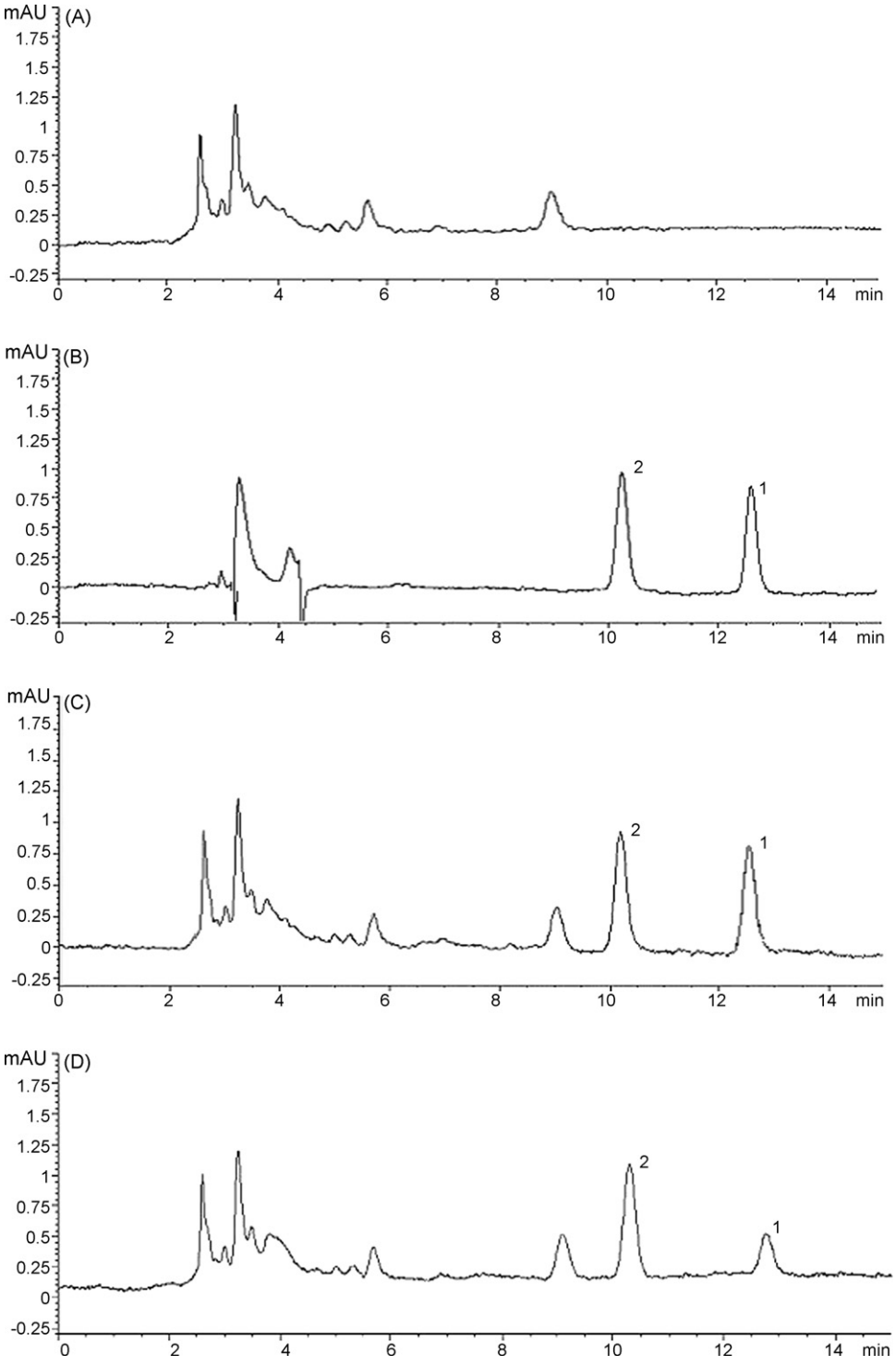

Fig. 2. Representative chromatograms of blank plasma (A); AL standard and I.S. standard in methanol (B); plasma spiked with AL (765 ng/ml) and I.S. (metronidazole, 500 ng/ml) (C); plasma samples obtained 1.5 h (D) after a single i.p. treatment with 10 mg/kg of AL from rats. Peak 1: AL; peak 2: metronidazole.

were then deproteinized with methanol. Nine samples were analyzed for each concentration. The absolute recoveries of AL were determined by comparing peak areas of extracted QC samples with those of corresponding concentrations standard solutions. The analysis was performed for three replicates at the concentration levels mentioned above. The mean recoveries of AL from rat plasma at concentrations of 114.8, 765.5 and 2449.6 ng/ml were 91.8, 93.9 and 96.0%. Using the same method, the recovery of I.S. in rat plasma was obtained which was 92.7%.

3.4. Sample stability

Stock solution of AL in methanol was found to be stable for at least 2 months when stored at 4 °C. The stability of AL

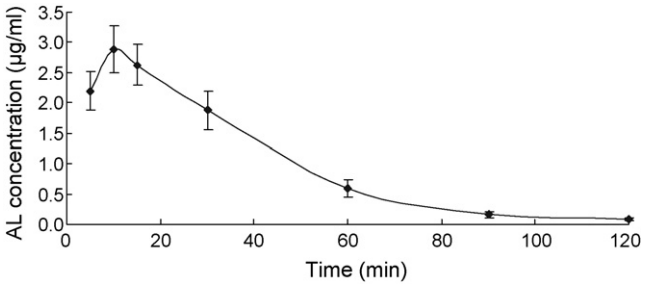

Fig. 3. Mean plasma concentration–time profile of AL after the i.p. administration of 10 mg/kg of AL to SD rat. Each point represented the mean of the concentration obtained from five rats.

in QC samples was not less than 10 days and AL was stable in plasma sample for at least 7 days when stored frozen at −20 °C (R.E. < −3.22%). Extracted plasma samples were found to be stable for at least 24 h when the samples were kept at room temperature (22–25 °C) and the final concentration were 93.8–98.2% of the initial values. AL was also stable following three freeze-thaw cycles. AL extracted from rat plasma samples in the mobile phase was stable with relative errors less than −4.2% for at least 24 h at room temperature.

3.5. Application of the method

The mean plasma concentration–time profile of AL in the plasma of SD rats following i.p. administration is shown in Fig. 3. With LOQ of 57.4 ng/ml, plasma concentrations of AL in pharmacokinetics study were successfully quantified by the assay up to 2 h (concentrations in 3 h samples were below LOQ). The basic pharmacokinetics parameters of AL in the rats were determined using DAS software. After i.p. administration. The drug disappears from the plasma according to a one compartment open model. The main pharmacokinetics parameters of AL are summarized in Table 2. The parameters suggested that AL cleared from plasma quickly. The details of the pharmacokinetics study will be described in a further publication.

3.6. Robustness of method

The result regarding the robustness of this method showed that the chromatographic patterns were not significantly changed when different solvent sources and a different HPLC system (Shimadzu, Japan) were used. Identical chromatograms were achieved when different analytical columns (Kromasil C18 column and Diamonsil C18 column) were used. In addition, this method allowed variation in analytical parameters such as pH adjusted to 2.4–3.5, acetonitrile content in the mobile phase variated between 11 and 8%, column temperature adjusted to 20–40 °C.

Table 2
Pharmacokinetics parameters for AL in rats after i.p. administration (10 mg/kg, $n = 5$)

Parameter result	Result (mean ± S.D.)
$T_{1/2}$ (min)	34.5 ± 2.32
T_{max} (min)	11.6 ± 0.81
C_{max} (μg/ml)	2.96 ± 0.44
CL/*F* (l/min/kg)	0.093 ± 0.005
Vz/*F* (l/kg)	3.58 ± 0.61
AUC (0–*t*) (mg/l min)	112.7 ± 7.46
AUC (0–∞) (mg/l min)	113.7 ± 6.95
K_e (1/min)	0.040 ± 0.008

4. Discussion

Wang assayed AL in aqueous humor using fluorospectrophotometry [12]. But the interference of endogenous substance to the determination of AL cannot be removed in this method. According to literatures and physicochemical characteristics of AL, we chose acetonitrile–0.1% triethylamine solution (adjusted to pH 3.0 using phosphoric acid) (10:90, v/v) as the mobile phase, and metronidazole as the internal standard (I.S.). AL, 7-hydroxy-coumarin-6-*O*-D-β-glycosides, is a coumarin glycosides. Enol form and lactone of the structure are easy to dissociate in alkaline conditions. Adjusting the pH of mobile phase to 3.0 can inhibit the dissociation of aesculin, prolong the retention time and improve peak shape remarkably. Metronidazole and AL exhibit marked absorbance at 338 nm. In the method we chose, I.S. presents a suitable retention time, good co-extraction (recovery >90% at the concentration used), and good resolution with AL. In addition, AL, I.S. and endogenous substance can be separated well using the described above method.

Plasma levels of AL are relatively low after single dose administration. For analysis of the drug using HPLC with UV detection, high efficient extraction procedures should be developed. Extraction efficacy of several organic solvents and saline solutions including methanol, acetonitrile, trifluoroacetic acid: acetonitrile (1:99), dichloromethane, $(NH_4)_2SO_4$ solution and $CuSO_4$ solution(1%) was investigated for extraction of AL from plasma. The highest extraction efficacy was obtained with methanol and no endogenous interference was found at the retention times of AL and the I.S. in chromatogram. The resolution between AL, I.S. and endogenous substances were more than 1.5, which met the requirements for quantification analysis.

Aesculetin and AL are the main effective constituents of *Cortex Fraxini*. These two compounds have coumarin as their parent structure. AL is the glucoside of aesculetin and easy to hydrolyze producing aesculetin and glucose in vitro. In our pharmacokinetics study of AL, aesculetin is not found in rats plasma sample after i.p. administration of AL The maximum absorbance of aesculetin is also 338 nm, therefore, using the method described in this article, aesculetin in plasma can be assayed simultaneously with AL following appropriate adjustment. Representative chromatograms for rat plasma spiked with AL, the I.S. and aesculetin using the HPLC method above are shown in Fig. 4.

Some related investigations indicated that the amount of AL will decrease and the amount of aesculetin will increase with the prolonging of the storage time of Cortex Fraxini in a damp environment. It is described in the Pharmacopoeia of the People's Republic of China that its curative effect decreases greatly when the content of AL in Cortex Fraxini was less than 1.36% [14,15]. Based on the fact and our finding, we suspected that AL may

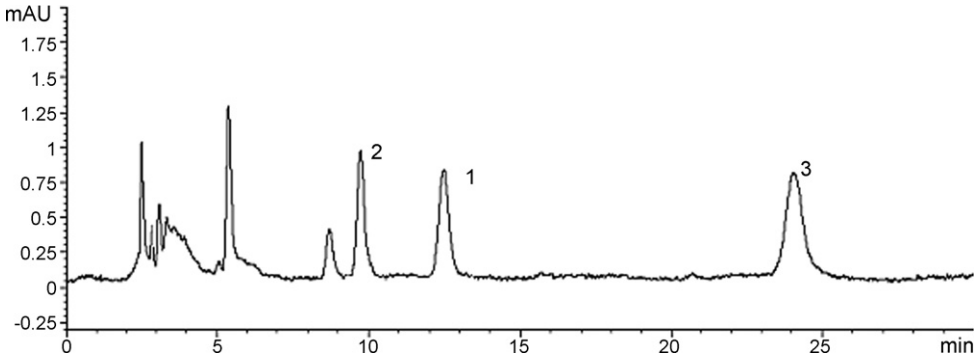

Fig. 4. Representative chromatograms of blank plasma spiked with AL, I.S. (metronidazole) and aesculetin. Peak 1: AL; Peak 2: metronidazole; Peak 3: aesculetin.

not be hydrolyzed to aesculetin in vivo. Because of relatively poor stability of AL, the plasma samples should be determined within 3 days.

5. Conclusion

In conclusion, a simple reversed-phase HPLC method has been described for the determination of AL in plasma. The method was characterized by a high degree of sensitivity, precision, accuracy and separation, and was capable to determine AL accurately down to 57.4 ng/ml. Due to the common instrument, easily obtained I.S., low LOQ and simple extraction procedure, the method is relatively practical. The method has been applied successfully for the first time to assay AL in plasma and to study in vivo pharmacokinetics of AL. In our future research, the chromatographic condition and sample preparation procedure in this article will likely to facilitate the development and validation of other HPLC methods to analyze AL in other biological matrixes such as urine and tissue homogenates.

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