Development of a Fluorescence Analysis Method for Determining Ampelopsin in Rabbit Plasma

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ABSTRACT: OBJECTIVE To establish a fluorescence analysis method for determining ampelopsin in rabbit plasma. **METHODS** After intravenous administration with ampelopsin at a single dose, the blood samples were collected and extracted with ethyl acetate. Ampelopsin concentrations were determined with fluorescence detection exciting at 442 nm and emission at 502 nm. **RESULTS** The calibration curve showed a linear response over the concentration range of 0.5–36.6 μg·mL^{-1} (r=0.999 6). The limit of detection was 70 ng·mL^{-1}. The recovery was between 98.78% and 103.57%. The relative standard deviations of intra-day and inter-day were less than 8.25%. **CONCLUSION** A rapid, simple and accurate method has been successfully applied to a pharmacokinetics study of ampelopsin in rabbits.

KEY WORDS: ampelopsin; fluorescence analysis; bioanalysis; pharmacokinetics

荧光分析法测定兔血浆中蛇葡萄素浓度

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摘要：目的 建立一种用于测定兔血浆中蛇葡萄素浓度的荧光分析方法。**方法** 经单剂量静脉给药后，收集家兔血样并经乙酸乙酯萃取，加入三氯化铝溶液后在激发波长 442 nm 和发射波长 502 nm 下进行血药浓度检测。**结果** 血浆中蛇葡萄素浓度在 0.5~36.6 μg·mL^{-1} 内与荧光强度呈良好线性关系，r=0.999 6。检测限为 70 ng·mL^{-1}，日内、日间精密度在 98.78%~103.57%之间，RSD 均小于 8.25%。**结论** 该方法简便、快速、准确，可用于蛇葡萄素的药代动力学研究。

关键词：蛇葡萄素；荧光分析法；生物分析；药代动力学

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Ampelopsin (dihydromyricetin), 3,3',4',5,5',7-hexahydroxy-2,3-dihydroflavanonol (shown in Fig 1), is extracted from Teng Cha (ampelopsis grossedentata) which is a popular plant in folk medicine in China. Potent biological effects have been described in many *in vivo* and *in vitro* studies[1]. Today, it has become components of many dietary supplements and medicine for these therapeutic properties.

But the studies mostly paid attention to potentially beneficial health effects of ampelopsin. Due to the low concentration of ampelopsin in plasma after administration with doses, it is critical to develop a sensitive analysis method. HPLC for determining ampelopsin in mice serum and plasma have been reported [2-3]. However, it is well known that the liquid chromatography techniques have many disadvantages including high expenditure, requiring excessive sample preparation and suffering from high complexity. Recently, human plasma samples have been directly determined by fluorimetry as reported [4]. It indicates that the inexpensive and efficient technique can objectively and reliably quantify drug levels in biological fluids.

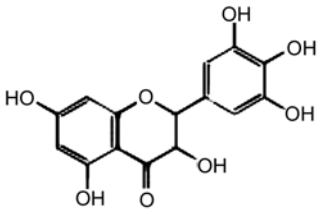

Fig 1 Chemical structure of ampelopsin
图 1 蛇葡萄素化学结构式

The present research aims to develop a sensitive, non-expensive and rapid method for the quantification of ampelopsin in rabbit plasma using fluorescence detection, and then to test its application in a pharmacokinetics study. Up to now, there are no similar methods published for determining ampelopsin in rabbit plasma.

1 Apparatus and reagents

1.1 Apparatus

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A Model 970CRT spectrofluorometer (Precision & Scientific Instrument Corporation, Shanghai, China) was used with excitation and emission slit widths of 5 nm, 3 orders sensitivity.

1.2 Chemicals and reagents

Ampelopsin standard was obtained from Zhangjiajie Shengli Biotechnology Co., Ltd, (Zhangjiajie, China). Aluminium trichloride, anhydrous ethanol, ethanol, polyethylene glycol (PEG-400), and dimethyl disulfoxide (DMSO) were purchased from Sinopharm Chemical Reagent Co., Ltd, (Shanghai, China). Ethyl acetate was purchased from Shantou Dahao Fine Chemicals Co., Ltd, (Shantou, China).

2 METHODS

2.1 Standard solutions

Ampelopsin standard solutions (2 mg·mL^{-1}) were prepared by dissolving ampelopsin in anhydrous ethanol, and then stored at 4 ℃ before using. The aluminium trichloride solutions (1%) were prepared by dissolving in ethanol, and then stored at room temperature. The ampelopsin solutions for intravenous administration were prepared by dissolving ampelopsin in mixed solvent (DMSO∶PEG-400∶physiological saline=5∶45∶50) with ultrasonic vibrating.

2.2 Sample preparation

Frozen plasma samples were thawed at room temperature. The plasma samples included blank plasma samples, quality control samples and pharmacokinetic plasma samples. Then, aliquots of 100 μL plasma samples were placed into heparinized tubes containing the volume of 400 μL acetate buffer solution (pH=4.5) with vibrating for 1 min. After adding the volume of 2 mL ethyl acetate with vibrating for 1 min, the samples were centrifuged at 3 000 r·min^{-1} for 10 min. Afterwards, the organic layer was used for determining ampelopsin by fluorimetric measurements.

2.3 Fluorescence measurements

Spectrophotometric studies were carried out in the emission wavelength range of 450–600 nm at the temperature of 25 ℃. To acquire measurable fluorescence, the samples were mixed with the volume of 1 mL aluminium trichloride solution (1%) in anhydrous ethanol with vibrating for 10 min[5].

2.4 Pharmacokinetic study

Six rabbits (weighing between 2.0–2.5 kg, ♂ ♀) were obtained from Fujian Health College (SYXK2006-0004). The rabbits were free access to food and water before processing the experiments. Ampelopsin was administered by intravenous route at a single dose of 100 mg·kg^{-1} at the weight of rabbit. Then the blood samples (0.5 mL) were collected from each rabbit via marginal ear vein at the time points of 0 , 3 , 5 , 12 , 24 , 30 , 45 , 60 min. Afterwards, the blood samples were immediately centrifuged at 10 000 r·min^{-1} for 10 min to remove aggregates and solids in order to obtain the plasma. Then the plasma samples were stored at –18 ℃ until analysis.

3 RESULTS AND DISCUSSION

3.1 Verification of fluorescent approach for determining ampelopsin in plasma

In order to generate measurable fluorescence, aluminium (Ⅲ) was used as a complexing ligand to form fluorescent complexes with ampelopsin. The parameters, including solvent selection, aluminium (Ⅲ) strength and ion interference were systematically optimized in the study [5].

The fluorescence spectrum of ampelopsin was unique and characterized by the presence of a very specific band at 502 nm, serving as a drug marker. As shown in Fig 2, the fluorescence spectrums of ampelopsin, blank plasma and their mixtures indicates that there is no fluorescence band in the range of 450–600 nm in plasma samples after the extract procedure. The signal can be used for quantitative determination of ampelopsin in plasma samples.

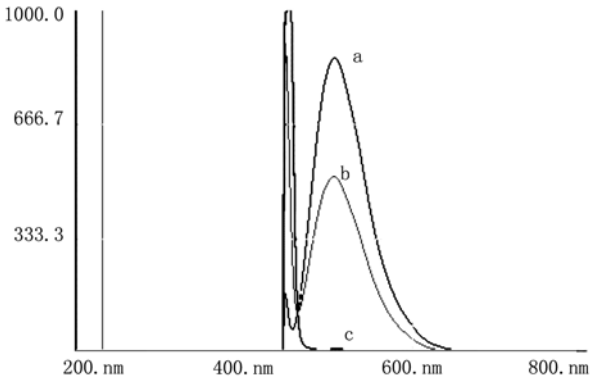

Fig 2 Fluorescence spectrum.

a–ampelopsin; b–blank plasma mixed with ampelopsin; c–blank plasma

图 2 荧光扫描图谱

a–蛇葡萄素；b–空白血浆+蛇葡萄素；c–空白血浆

3.2 Simplification of sample process

Protein precipitation is essential in the sample process. Three kinds of protein precipitation methods, such as ethanol, ammonium sulphate and non-precipitant, were compared by evaluating the effects of protein precipitation. Consequently, there was no significant difference among the methods. Thus, the method of non-precipitant was selected to deal with the protein in samples.

The amount of extracted analyte was relevant to extracting times. The effects of extracting times on the analytical signal were also investigated. The results indicate that the rate of multi-extraction was over 10% higher than that of a single extracting. However, the multi-extraction is complicated to manipulate and possible to cause excess error. Consequently, a single extracting was selected in this study.

The effects of residual extracted solvent were also investigated in this study by comparing the difference between the results before and after evaporating. Consequently, due to noninterference in the assay, ethyl acetate did not need to be evaporated.

The results show that the process of plasma samples was simplified and ensured a stable, reproducible and acceptable extraction rate (66.4 ± 3.56%).

3.3 Method validation

3.3.1 Linearity of calibration curves and limit of detection Calibration curves were investigated by replicate analyses of ampelopsin standard solutions with a series of concentrations by plotting the fluorescence intensity at 502 nm. Each test was done in triplicate. In conclusion, the calibration curve was found to be linear within the concentration range of 0.5–36.6 μg·mL^{-1} in rabbit plasma with correlation coefficient (r=0.999 6). The typical calibration curve is Y=15.42C–1.036(n=6). The results show that there was good linear relationship between the fluorescence intensity and concentrations of ampelopsin.

The limit of detection (LOD) was obtained by performing replicate analyses of a calibration solution with the lowest concentration (0.5 μg·mL^{-1}). LOD was calculated to be 70 ng·mL^{-1} on the basis of extrapolation to a signal-to-noise (S/N) ratio of 3∶1.

3.3.2 Precision and accuracy The precision and accuracy of the method were calculated from five replicate analyses of the plasma samples under the complete analytical procedure. The intra-day precision of assays were tested using three concentrations of ampelopsin (2.25, 9.00 and 36.00 μg·mL^{-1}). The inter-day precision was detected with the same procedure once a day for five consecutive days. Accuracy was determined by comparing the calculated concentration using calibration curve to nominal concentration.

The results of the accuracy and precision of intra-day and inter-day are shown in Tab 1. The inter-day accuracy and precision were studied over 5 days. The results show that the intra-day accuracy was between 102.89% and 104.58% under three concentrations with RSD lower than 5.38%. The inter-day accuracy was between 98.78% and 103.57% under three concentrations with RSD lower than 8.25%.

Tab 1 Accuracy and precision of ampelopsin in rabbit plasma (n=5, $\bar{x} \pm s$)

表 1 兔血浆样品中蛇葡萄素检测的准确度与精密度(n=5, $\bar{x} \pm s$)

Nominal concentration/μg·mL^{-1}	Intra-day precision			Inter-day precision		
	Measured concentration/ μg·mL^{-1}	Accuracy/%	RSD/%	Measured concentration / μg·mL^{-1}	Accuracy /%	RSD/%
2.25	2.33±0.09	103.56±4.18	4.04	2.28±0.19	101.26±8.35	8.25
9.00	9.26±0.50	102.89±5.54	5.38	8.89±0.64	98.78±7.29	7.38
36.00	37.65±0.89	104.58±2.49	2.38	37.28±1.99	103.57±5.52	5.33

3.3.3 Stability The stability of samples was evaluated after experiencing three freeze/thaw cycles and room temperature, respectively. Three replicates of control samples at concentrations of 2.25, 9.00 and 36.00 μg·mL^{-1} were mixed with blank plasma. Then, the mixing solutions were subjected to three freeze/thaw cycles or stored at room temperature for 24 h before sample processing.

The results in Tab 2 show the stability of ampelopsin in rabbit plasma under different storage

conditions, including at room temperature and –18 ℃. After experiencing three freeze/thaw cycles or being stored at room temperature for 24 h, the deviation of ampelopsin concentrations in plasma was less than 2.36% in comparison with the reference nominal concentrations. It indicates that there was no significant degradation in rabbit plasma samples during the process. Therefore, the plasma samples were stable under routine laboratory conditions.

In addition, the fluorescent stability of samples after adding aluminium (Ⅲ) had been investigated at room temperature. By comparing the variety of fluorescence intensity during 24 h, the results show that the intensity kept constant within 10 min, and thereafter continually decreased to 60%[5]. Therefore, the samples should be assayed in 10 min.

Tab 2 Stability of ampelopsin in rabbit plasma (n=5, $\bar{x} \pm s$)

表 2 血浆中蛇葡萄素的稳定性试验(n=5, $\bar{x} \pm s$)

Spiked concentration /μg·mL^{-1}	Three freeze/thaw cycle stability (–18 ℃)		Post-preparative stability (24 h at room temperature)	
	Mean	RSD/%	Mean	RSD/%
2.25	2.27±0.04	1.65	2.27±0.07	2.87
9.00	9.12±0.19	2.11	8.91±0.10	1.13
36.00	36.61±0.86	2.36	36.77±0.45	1.23

3.4 Pharmacokinetic study

The developed and validated method was applied to the pharmacokinetic study of ampelopsin in rabbits. According to the maximum of r^2 and the minimum of AIC and GOF, the pharmacokinetic parameters were calculated by 3P87 program (Practical Pharmacokinetic Program, 1987, China) and showed in Tab 3. The results in Fig 3 show that the curves of ampelopsin concentration in plasma versus time were fitted to two-compartment model.

Tab 3 Criteria for Goodness of fitting

表 3 主数据拟合优度用于模型判断

Weighting Factor	Compartment	r^2	GOF	AIC	WSS
1	1	0.975 9	1.307 8	15.537 8	6.841 2
1	2	0.999 3	0.306 2	–2.042 0	0.187 6
1/C	1	0.996 6	0.486 2	3.664 0	0.945 5
1/C	2	0.999 6	0.249 7	–4.489 6	0.124 7
1/C/C	1	0.999 4	0.213 5	–6.210 8	0.182 4
1/C/C	2	0.999 7	0.205 7	–6.816 3	0.084 6

Note: GOF=Goodness of fitting; AIC=Alkake's Information of Criterian; WSS=weighted surplus sum of squares

注：GOF为拟合优度；AIC为赤池信息量准则；WSS为加权残差平方和

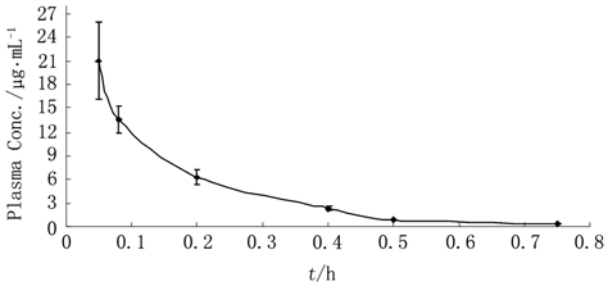

Fig 3 Mean plasma concentration-time profiles of ampelopsin in rabbits after intravenous administration at a dose of 100 mg·kg^{-1} at weight of rabbit(n=6, $\bar{x} \pm s$)

图 3 家兔静脉给药后血药浓度-时间曲线图(n=6, $\bar{x} \pm s$)

After intravenous administration of ampelopsin, the values of pharmacokinetic parameters were estimated by 3P87 program and depicted in Tab 4. The total body clearance (CL) was 22.63±1.78 L·(kg·h)$^{-1}$. And the area under the plasma concentration-time curve (AUC) was 4.37± 0.42 μg·h·mL^{-1}. In addition, distribution half life ($t_{1/2\alpha}$) was obviously smaller than elimination half life ($t_{1/2\beta}$), indicating that ampelopsin distributed widely in the rabbit tissues. In summary, the method could be successfully applied to the pharmacokinetics study of ampelopsin in rabbits.

Tab 4 Pharmacokinetics parameters of ampelopsin in rabbits after intravenous administration at a dose of 100 mg·kg^{-1} body weight(n=6, $\bar{x} \pm s$)

表 4 蛇葡萄素在家兔体内的药动学参数(n=6, $\bar{x} \pm s$)

Parameter	Unit	Value
A	μg·mL^{-1}	57.21±2.52
B	μg·mL^{-1}	14.75±0.68
α	1·h^{-1}	41.43±2.38
β	1·h^{-1}	5.61±0.33
V(c)	L·kg^{-1}	1.27±0.23
$t_{1/2}\alpha$	H	0.019±0.003
$t_{1/2}\beta$	H	0.14±0.02
K_{21}	h^{-1}	12.75±1.03
K_{10}	h^{-1}	17.02±1.35
K_{12}	h^{-1}	16.37±1.61
$AUC_{0\text{-}0.75h}$	μg·h·mL^{-1}	4.37±0.42
CL(s)	L·(kg·h)$^{-1}$	22.63±1.78

4 CONCLUSIONS

In this study, the novel fluorimetric method of quantitative determination of ampelopsin has been successfully developed and applied to the pharmacokinetics study in rabbits. The method has the virtue of being inexpensive and efficient, and will be implemented objectively and reliably in any

laboratory. It is expected that the method will be further used in analyzing biological samples.

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