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Short communication

The experimental study of *Cortex Eucommiae* on meridian tropsim: The distribution study of aucubin in rat tissues

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Abstract

Meridian tropism (MT) theory is a core principle of traditional Chinese medicine (TCM) theories and plays an essential role in instructing clinical pharmacy. The scientific explanation of MT theory will certainly further promote the reasonable, effective application of TCM. In view of the MT of *Cortex Eucommiae* (CE), aucubin (AU), the effective component of CE, was appointed and observed its distribution in rat tissues following a single intravenous (i.v.) dose. A simple, inexpensive and accurate high-performance liquid chromatographic (HPLC) method was developed and validated for the determination of AU in rat tissues. Acceptable intra-day and inter-day precision and accuracy at high, medium and low concentration ranged from 0.56% to 4.18% and 0.73% to 4.53%, respectively. Good assay and extraction recoveries were obtained with a single and relatively fast precipitation protein step. The mean assay recovery and extraction recovery of AU were 94.7% and 90.9%, respectively. All tissues reached maximum AU level at 5 min post-dose. Considerable AU was present in kidney and liver. AU concentration was highest in kidney and remained much higher than that in other tissues over the experiment course. Lung, heart, spleen and testis were also detected to contain AU. The results closely conformed to the MT of CE and clearly demonstrated that AU was one of the material bases of the MT of CE.

Keywords: Meridian tropism; *Cortex Eucommiae*; Aucubin; Tissue distribution

1. Introduction

MT theory is a core principle of TCM theories and plays an essential role in clinical selection of TCM according to syndromes, raising the specificity of medicine and strengthening the therapeutic effects. It takes the theory of viscera, meridians and the indication of syndromes as basis, exhibits the selectivity of drug therapeutic effects. MT means that Chinese traditional herbs may act on some portion of human body in preference. In another word, the meridians are just related to the certain meridians on which TCM may work. The scientific explanation of MT theory will certainly further promote the reasonable, effective application of TCM. At present, the experimental methods for studying this theory are mainly observation of the active compound and microelements distribution *in vivo*, the pharmacological efficacy and the variation of the cyclic adenosine monophosphate and the cyclic guanosine monophosphate [1,2]. The active compound is the material basis of drug therapeutic effects, so studying MT through the compound distribution *in vivo* is conducive to clarifying the mechanism and the selectivity of drug actions in essence. CE, a top-grade medicine considered at "Shennong's Herbal Classics", has antihypertensive, antiinflammatory, antiviral, invigorating the kidney activities and is famous on its high-quality hepatoprotective activity without side effects [3–5]. CE contains mainly iridoid, lignan, flavonoid compounds and so on. The experimental study of CE on MT is rarely reported.

Pharmacological studies showed that the iridoid glucosides, particularly AU, had diverse biological activities and possessed remarkable hepatoprotective effect. AU (Fig. 1), abundant and widespread in nature, is the principal and abundant component of CE and also characteristic for many species of *Plantago* and *Veronica*. It exhibited significant liver-protective activity against carbon tetrachloride [6] and α-amanitin-induced hepatotoxicity in mice [7]. Conversion to its aglycone form appeared to be a prerequisite step for the inhibition of ethoxy coumarin *O*-

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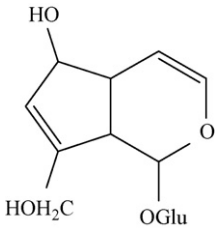

Fig. 1. Structure of AU.

deethylase [8] and antiviral activity [9]. AU also inhibits the releasing of leukotriene C4 and thromboxane B2 from calcium ionophore-stimulated samples [10] and the activation of nuclear factor-kappa B in the mast cells, which might explain its beneficial effect in the treatment of chronic allergic inflammatory diseases [11,12]. Recently, AU was found to be a novel topoisomerase I inhibitor which increased interest in its possible use in cancer chemoprevention and antitumoral therapy [13]. Other biological activities of this natural compound, such as promoting collagen synthesis [14,15], photoprotective activity [16] and trypanocidal potential against *Trypanosoma brucei rhodesiense* [17] were also observed.

Although AU exhibited a variety of important pharmacological activities, there was only limited information in the literature regarding its pharmacokinetics. Suh and colleagues had previously investigated the pharmacokinetics and bioavailability of AU administered i.v., orally, intra-peritoneally and hepatoportally to rats [18]. Distribution of radioactivity in the liver and kidney were examined in rats at 4 h after oral or i.v. 100 mg/kg dosing of [^{3}H]AU [19]. To the best of our knowledge, there has not been reported on the kinetic distribution of AU in rat tissues. The experiment described here aimed to study the conformance of the tissue distribution of AU and the MT of CE, and accumulate scientific evidences for explanation the position and essence of meridian.

2. Experimental

2.1. Chemicals and reagents

AU, white crystalline powder, was isolated from the seeds of *Eucommia ulmoides Oliv*. which were purchased from Jiahe Duzhong Industry Co. Ltd. (Lueyang, China). Biomedical Key Laboratory of Northwest University authenticated the crystalline powder. HPLC-grade acetonitrile and methanol were purchased from Tedia (Fairfield, USA). HPLC-grade water was obtained using the Millipore Simplicity water purification system (Millipore S.A.S. 67120Molsheim, France). Solvents for HPLC were thoroughly degassed in an ultrasonic bath before use. All other reagents were of analytical grade and were used without further purification.

2.2. Animals

Male Sprague Dawley rats (245–270 g) were purchased from Shaanxi Academy of TCM (Xian, China). Animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the State Food and Drug Administration. All animals were housed under standard conditions and had ad libitum access to water and a standard laboratory diet for at least 3 days except for the final 12 h before experiment.

2.3. Instrument and chromatographic conditions

A HPLC (Waters-Alliance, USA) system was composed of a 2695 separation module and a 2487 dual wavelength absorbance detector, which were controlled by Empower software. Separation was performed on a reverse-phase Diamonsil C18 column (5 μm, 250 mm × 4.6 mm i.d., Dikma) at 30 ± 1 °C. HPLC conditions were optimized to obtain maximum sensitivity. The isocratic mobile phase was water–acetonitrile (97.2:2.8, v/v) for analyzing the liver, heart, spleen, testis samples and water–acetonitrile (96.9:3.1, v/v) for analyzing the kidney and lung samples. The mobile phase was filtered through a 0.20 μm membrane filter to remove any particulate matter and degassed by ultrasonic wave before use. The analysis was performed with a flow rate of 1.0 ml/min and UV detection at 206 nm. The sample injection volume was 10 μl.

2.4. Sample preparation

In a 1 ml micro-centrifuge tube, 100 μl supernatants of tissue homogenates alone with 30 μl zinc sulfate solution (15% w/v) and 70 μl methanol were added to precipitate tissue protein. After vortexing for 1 min using an electrical shaker, the mixtures were centrifuged at 9000 × *g* for 5 min. The supernatant transparent liquids were transferred to auto-sampler microvials for injection and analysis. Samples with drug concentrations above the calibration range were diluted with saline prior to sample extraction.

2.5. Standard solutions

An AU stock solution (1.0 mg/ml) was prepared in methanol and stored at −20 °C. Varying volumes of AU stock solutions were added to tubes, and the solvent was evaporated. Residues were serially diluted with supernatants of homogenates to 1, 5, 20, 50, 100, 150, 200 μg/ml solutions for the liver, 1, 5, 20, 50, 100, 200, 400 μg/ml solutions for the kidney and 0.5, 1, 5, 10, 20, 50, 100 μg/ml solutions for other tissues. Quality control (QC) solutions of AU (5, 20, and 100 μg/ml) were independently diluted.

2.6. Method validation

Six complete sets of AU standards were analyzed. These included standards with concentrations ranging from 1 to 200 μg/ml in liver, 1 to 400 μg/ml in kidney and 0.5 to 100 μg/ml in other tissues. The calibration curves were generated by least-squares linear regression of the peak area versus AU concentration. The QC samples at low, medium and high concentrations and the limit of quantitation (LOQ) samples were analyzed to determine the accuracy and precision of the proposed method. Intra-day and inter-day accuracy and precision

were determined over a period of three consecutive days with five replications at each concentration per day ($n = 15$). The precision was evaluated as the relative standard deviation (%, R.S.D.). The accuracy (%, mean ± S.D.) was calculated by comparing the concentrations of AU from the calibration curves with that in the prepared sample. The LOQ was defined as the lowest concentration on the standard curve that can be discriminated from the baseline level with signal intensity at least ten times greater than the background level. The limit of detection (LOD) was considered to be the lowest concentration that can be discriminated from the baseline level with signal intensity at least three times greater than the background level. The extraction recoveries were determined by comparing the responses of AU extracted from replicate QC samples with the responses of AU from non-extracted standard solutions at equivalent concentrations.

2.7. Application to study the distribution of AU

Animals were killed by exsanguinations at 5, 10, 30, 45, 60, 90, 120, 180 and 240 min after administration 25 mg/ml AU, 100 mg/kg body weight, via the femoral vein. The heart, kidney, liver, lung, testis and spleen tissues were rapidly dissected and washed off blood with ice-sterilized physiological saline. These tissues were gently blotted with absorbent paper, weighted and sliced coarsely with scissors. The tissues were added to ice-cold sterilized physiological saline (50% tissue w/v) and homogenized in a homogenizer. After transferred to ultracentrifuge tubes, the homogenates were centrifuged at $9000 \times g$ for 5 min and the supernatants were separated and stored at −20 °C until analysis. The tissue samples were prepared and measured for AU concentrations as described above. The mean

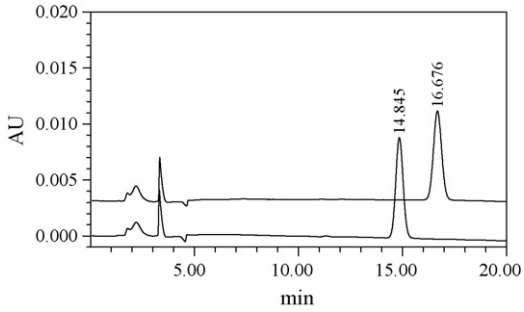

Fig. 2. Chromatograms of AU at the concentration of 50 μg/ml when the ratio of water:acetonitrile were at 97.2:2.8 (top) and 96.9:3.1 (bottom) levels.

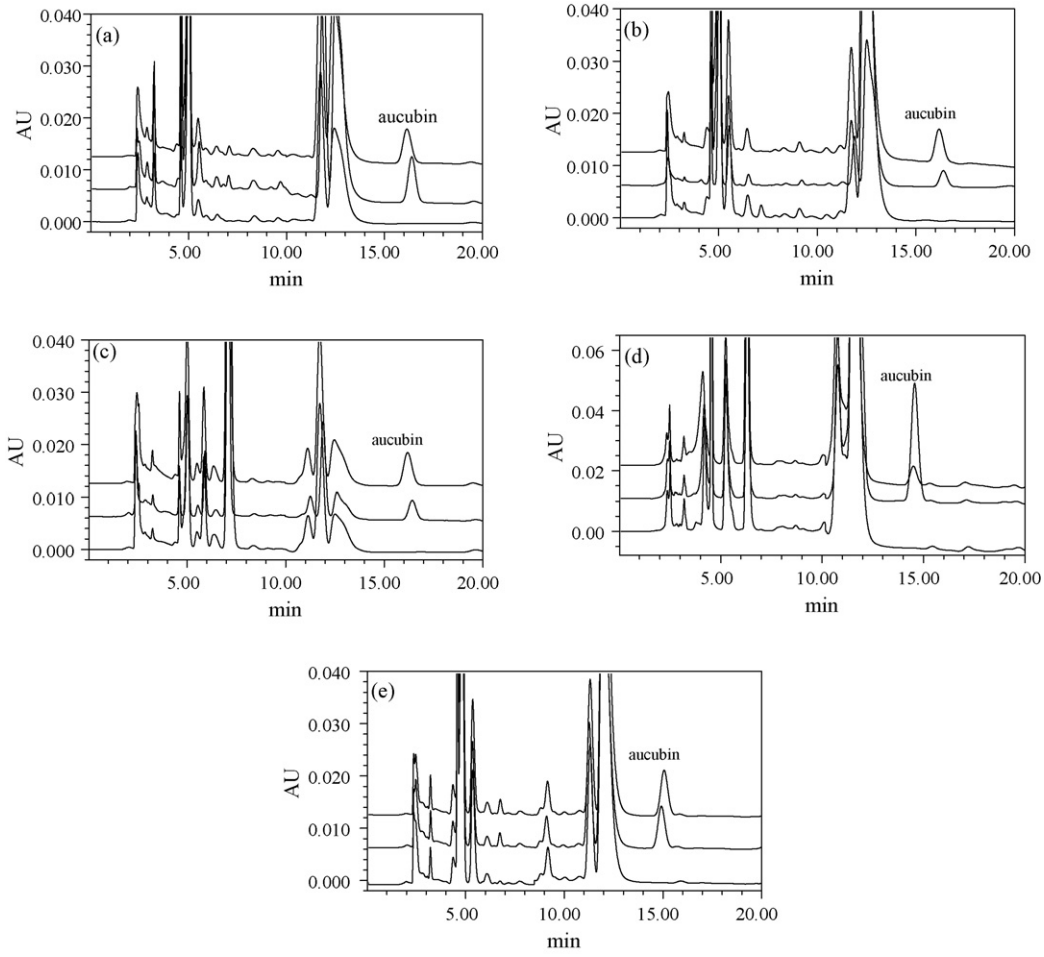

Fig. 3. Chromatograms of rat blank tissue, tissue sample obtained at 30 min after i.v. administration and blank tissue spiked with AU at the concentration of 50 μg/ml (from bottom to top): (a) liver, (b) spleen, (c) heart, (d) kidney and (e) lung.

tissueconcentrations of AU versus time data were analyzed using DAS program (2005). Area under the concentration curve (AUC_{0-x}, with x being the time of the last tissue concentration measured) was estimated using the linear trapezoidal method. Area under the tissue concentration curve to infinity ($AUC_{0-\infty}$) was estimated by dividing the value of the last tissue concentration measured by the terminal tissue rate constant. The half-life of the elimination phase ($t_{1/2\beta}$) and the clearance rate (CL) were also calculated from tissue concentration data.

3. Results and conclusion

3.1. Chromatographic procedure

The representative chromatograms of AU (purity of 99.3%) standard obtained at the two mobile phase conditions were shown in Fig. 2. The blank tissue, tissue sample spiked with AU and a tissue sample obtained at 30 min from a rat following i.v. administration of AU (100 mg/kg) were shown in Fig. 3a–e for liver, spleen, heart, kidney and lung. The retention times of AU were 14.84 and 16.67 min when the ratios of water-acetonitrile were at 97.2:2.8 and 96.9:3.1 levels, respectively. The peak of AU in sample was identified by comparing its retention time with that of the standard. The HPLC method achieved good baseline separation of AU with tissue endogenous compounds.

In this study, several organic solvents were tested for precipitation of protein and extraction of AU from tissues. They included methanol, ethanol, acetonitrile, ethyl acetate, *n*-butyl alcohol and their volume were triple that of homogenates. The result showed that the effect of precipitating protein and extraction recovery were best when methanol and acetonitrile were chosen, and the HPLC chromatogram was best when methanol was chosen. In order to enhance the detection sensitivity, zinc sulfate solution was used to precipitate protein and reduce the volume of methanol.

Table 1
Calibrations for analysis of AU in rat tissues ($n = 5$)

Tissue	Slope	Intercept	Range (μg/ml)	γ
Liver	3875	−370	1–200	0.9998
Kidney	3826	−209	1–400	0.9999
Heart	3837	1445	0.5–100	0.9998
Lung	4176	1094	0.5–100	0.9997
Spleen	3968	425	0.5–100	0.9997
Testis	3805	1590	0.5–100	0.9997

3.2. Assay validation

The current assay allows the quantification of AU over a wide concentration range as shown in Table 1. Standard curves generated from different ranges of AU concentrations in different tissues demonstrated acceptable linearity with $\gamma > 0.9995$ at concentration ranging from 1 to 200 μg/ml in liver, 1 to 400 μg/ml in kidney and 0.5 to 100 μg/ml in other tissues. The LOQ and LOD were 0.5 and 0.1 μg/ml for AU, respectively, with R.S.D. both less than 5% ($n = 6$). These were considered acceptable for the tissue distribution study. Precision and accuracy data confirmed the good reproducibility of the described method in Table 2. The inter- and intra-day precision ranged from 0.56% to 4.18% and 0.73% to 4.53%, respectively. Good assay and extraction recoveries were obtained with a single and relatively fast precipitation protein step. The mean assay recovery and extraction recovery of AU were 94.7% and 90.9%, respectively. The method was shown to be accurate for all tissues.

3.3. Stability

The QC samples showed no significant degradation after three freeze–thaw cycles. In extracts, AU was stable for up to 48 h

Table 2
Precision, accuracy and recovery for the determination of AU in rat tissues ($n = 5$)

Tissue	Conc. spiked (μg/ml)	Inter-day (%)		Intra-day (%)		Extraction recovery (%) (mean ± S.D.)
		Precision (R.S.D.)	Accuracy (mean ± S.D.)	Precision (R.S.D.)	Accuracy (mean ± S.D.)	
Liver	5	3.23	88.5 ± 3.26	3.52	85.7 ± 3.46	88.9 ± 3.13
	20	0.95	98.4 ± 0.96	2.04	96.1 ± 2.02	89.8 ± 1.83
	100	1.83	97.0 ± 1.79	2.38	96.1 ± 2.30	92.2 ± 2.20
Kidney	5	4.18	92.0 ± 4.09	3.56	93.0 ± 3.51	87.7 ± 3.12
	20	0.56	97.7 ± 0.56	1.12	97.4 ± 1.11	91.0 ± 1.02
	100	1.69	96.5 ± 1.63	1.31	97.0 ± 1.27	93.2 ± 1.22
Lung	5	2.85	92.1 ± 2.77	3.88	88.1 ± 3.62	90.4 ± 3.51
	20	0.94	98.4 ± 0.93	2.42	96.0 ± 2.35	94.3 ± 2.28
	100	1.20	97.6 ± 1.18	3.15	94.2 ± 2.98	94.1 ± 2.97
Heart	5	2.24	92.9 ± 2.25	2.65	90.4 ± 2.59	87.2 ± 2.31
	20	0.76	97.5 ± 0.76	0.94	97.0 ± 0.93	89.1 ± 0.84
	100	0.84	98.8 ± 0.84	1.42	97.7 ± 1.40	92.0 ± 1.31
Spleen	5	3.29	93.8 ± 3.16	4.53	91.0 ± 4.22	90.0 ± 4.07
	20	0.94	94.9 ± 0.90	0.73	94.9 ± 0.70	91.0 ± 0.66
	100	1.75	96.7 ± 1.69	2.01	94.9 ± 1.91	92.1 ± 1.86
Testis	5	1.83	87.0 ± 1.74	3.92	88.9 ± 3.81	90.0 ± 3.53
	20	0.96	97.8 ± 0.96	1.41	97.3 ± 1.40	90.9 ± 1.28
	100	1.34	98.0 ± 1.32	2.00	98.9 ± 2.00	92.3 ± 1.86

Conc.: concentration.

Table 3
Kinetic parameters of AU in tissues

Parameter	Unit	Tissue					
		Kidney	Liver	Lung	Heart	Spleen	Testis
$t_{1/2\beta}$	min	42.63	64.44	69.32	69.32	69.32	69.32
CL	l/(h kg)	0.24	0.72	1.08	1.86	2.46	2.64
AUC_{0-x}	min mg/ml	23.94	7.52	4.57	2.59	1.95	1.90
$AUC_{0-\infty}$	min mg/ml	26.91	8.64	5.51	3.18	2.41	2.29

at ambient temperature. Also, there was no significant difference in tissues after storage at −20 °C for 3 days. Stock methanol solutions of AU were stable for up to 10 days at 4 °C.

3.4. The distribution of AU in tissues

To study the MT of CE, the distribution of AU in rat tissues was measured after i.v. administration of 100 mg/kg AU. The major pharmacokinetic parameters were listed in Table 3. The concentration–time profiles in different tissues were shown in Fig. 4a and b. AU was widely distributed to tissues after a single i.v. dose. It has been reported that the small volume of distribution of AU indicated that this compound is poorly distributed to body tissues [18]. In this study, AU could be detected in many tissues including blood-abundant tissue (i.e., kidney, liver, heart, lung, spleen) and blood-rare tissue (i.e., testis). Of all examined tissues, the kidney had the highest AU concentration, followed by the liver, lung, heart, spleen and testis. The original concentrations in tissues at 5 min post-dose were 511.3 ± 42.03, 91.3 ± 11.29, 98.4 ± 6.40, 77.4 ± 5.62, 46.8 ± 5.44 and 34.7 ± 3.55 ug/ml (mean ± S.D., $n = 6$). The concentrations of AU in liver were higher than those in lung at all other time point except for the origination. The AUC_{0-x}, of AU

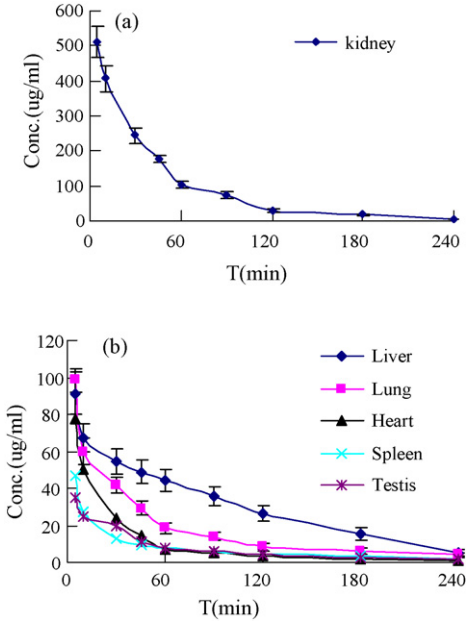

Fig. 4. Concentration–time profiles of AU in rat tissues. Each time point represents the mean ± S.D. of six independent trials: (a) kidney and (b) liver, lung, spleen, heart and testis.

in kidney and liver were 23.94 and 7.52 min mg/ml, respectively, and much larger than that in other tissues. The high content of AU in the kidney might be a reflection of the rapid renal elimination after i.v. administration. The last concentrations of AU in all tissues were lower than 6 ug/ml. Therefore, renal elimination may be a major elimination pathway for AU. As might be predicted in light of the hypotoxic nature of AU, the half-life in tissues was short.

4. Conclusion

A HPLC method for determination of AU in rat tissues has been developed for the first time. It involved a simple one-step protein precipitation and was less time-consuming and tedious than other reported methods. Our method has acceptable sensitivity, precision, accuracy, selectivity, recovery and stability, and has been successfully applied to the tissue distribution study of AU after i.v. administration of AU to rats.

The MT theory was first documented in the TCM classic book "Emperor's Inner Classic" (Huangdi Nei Jing). This classic Chinese manuscript defined the meridian as "the determining factor of life and death, helps to maintain health verses disease and regulates the balance of deficiency and excess". In accordance with this theory, CE was applied for invigorating the liver and kidney, strengthening the tendons and bones, was attributed to the liver and kidney meridians. This experimental study showed that AU, the key active constituent of CE, was widely and rapidly distributed to tissues following a single i.v. administration and mainly distributed to the kidney and liver. This result conformed to the MT of CE, so it clearly demonstrated that AU was one of the material bases of the MT of CE. Although the modern scientific definition of meridian is not completely clear, in some extent, the experimental study of observing the active compound distribution in tissues can help to accumulate the scientific data for the explanation the position and essence of meridian.

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