doi.org/10.36721/PJPS.2020.33.3.REG.953-960.1

Investigation on the urinary excretion kinetics of three atractylenolides from crude and processed *Atractylodis rhizoma* extracts in rats by UPLC-MS/MS

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Abstract: *Atractylodis rhizoma* is a frequently-used traditional Chinese medicine in clinical practice, which have the effect of eliminating dampness and tonifying spleen. And after being processed with wheat bran, the dryness of *A. rhizoma* is reduced, and the function of tonifying spleen is enhanced. Atractylenolides are the major bioactive components of *A. rhizoma*, including atractylenolide I (AI), atractylenolide α (Aα) and atractylenolide β (Aβ). The present study aimed to develope a new UPLC-MS/MS method for simultaneous quantification of three atractylenolides in rat urine, and applied to the excretory kinetics in Sprague-Dawley rats after oral administration of crude and processed *A. rhizoma* extracts. Analytes and internal standard were detected without interference in the multiple reaction monitoring (MRM) mode with positive electrospray ionization. The excretory kinetics parameters were calculated by a urine drug analysis model of drug and statistics (DAS) 3.2.8 software. The $t_{1/2}$ and Ke of three atractylenolides had no significant difference between crude and processed *A. rhizoma*, but the recovery accumulative excretion of them in processed *A. rhizoma* were apparently higher than the crude ones ($p<0.05$, $p<0.01$). The results showed that only a small amount of atractylenolides excreted in urine and processing *A. rhizoma* with wheat bran by stir-frying could promote the urinary excretion of them.

Keywords: *Atractylodis rhizoma,* atractylenolide, UPLC-MS/MS, excretion, stir-frying with wheat bran.

INTRODUCTION

Traditional Chinese medicine (TCM) have been widely used for thousands of years in China, obtaining a growing acceptance in the international medical field, nowadays. Therefore, the modernization of TCM and natural products have become indispensable. *Atractylodis rhizoma* (Cangzhu in Chinese) is the dried rhizome of *Atractylodes lancea* (Thunb.) DC. or *Atractylodes chinensis* (DC.) Koidz. of Asteraceae, which was first recorded in the ancient pharmaceutical classic "Sheng Nong's herbal classic". Herb has been used since far-back time to treat rheumatic diseases, digestive disorders, mild diarrhea and influenza (China 2015). There were many complex chemical components in *A. rhizome* ,which were detected by modern analytical techniques, including volatile oils, polyacetylenes, sesquiterpenoids, lactones, and polysaccharides (Ouyang *et al.*, 2007; Ji *et al.*, 2001; Chen *et al.*, 2015; Xu *et al.*, 2016; Xu *et al.*, 2017). Modern pharmacological studies showed that *A. rhizoma* have the effects of anti-inflammatory, antimicrobial, anti-ulcer, hypoglycemic and hepatoprotective (Koonrungsesomboon *et al.*, 2014, Wang *et al.*, 2009, Yu *et al*., 2015, Niu, 2014, Xu *et al.*, 2016; Na-Bangchang, 2014). In the TCM theory, *A. rhizoma* is often processed by stir-frying with wheat bran with the aim of reducing its dryness and increasing the function of tonifying spleen, while some studies also indicated that the content of multiple components had changed after processed (Wang *et al.*, 2007; Xu *et al.*, 2007).

The pharmacokinetic research of TCM is an inalienable part of the TCM modernization and plays an important role in the TCM novel drug development. Our research group had measured the difference of pharmacokinetics and tissue distribution of some effective ingredients between crude and processed *A. rhizoma*, in previous studies (Huo *et al.*, 2014; Chang *et al.*, 2016; Liu *et al.*, 2017). The results preliminarily showed that processed *A. rhizoma* could promote the absorption *in vivo*. So far, however, there have been no reports of studies on excretion of *A. rhizoma*. The excretion of drugs is closely related to the duration of drug action and the adverse reaction *in vivo*. And comparing with plasma and tissue, the change of urine drug concentrations are more likely to elucidate the elimination process of drugs *in vivo*. Therefore, we would study further on the excretory regularity of crude and processed *A. rhizoma*.

Atractylenolides are the major bioactive compounds of *A. rhizoma*, including atractylenolide I (AI), atractylenolide α (Aα) and atractylenolide β (Aβ). Modern pharmacological have carried out extensive studies on atractylenolides, found out that these components had anti-inflammatory (Ji *et al.*, 2014; Yoou *et al.*, 2017; Ji *et al.*, 2016) and anti-tumor (Wang *et al.*, 2006; Liu *et al.*, 2016; Fu *et al.*, 2014) activity. Moreover, recent researches also showed that A could improve the function

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of blood coagulation and had potential applications in the treatment of leukemia (Tang *et al.*, 2017; Huang *et al.*, 2016). There were many literatures reported the studies of pharmacokinetics and tissue distribution in rats of atractylenolides (Li *et al.*, 2006; Ge *et al.*, 2007; Wang *et al.*, 2008; Yan *et al.*, 2015), but most of them investigated with a single substance administration, and the record about the comparisons of these compouds between crude and processed *A. rhizoma* is deficient so far.

The present paper aimed to develop a new UPLC-MS/MS method for the quantification of three atractylenolides in rat urine and applied to the study of excretion kinetics. We would preliminary clarify the excretory regularity of atractylenolides after oral administration and the excretory difference between crude and processed *A. rhizoma.* This research would be necessary and helpful for further studies on the processing mechanism and clinical safety.

MATERIALS AND METHODS

Laboratory animal

Twelve healthy adult male Sprague-Dawley rats, weight 180 ～ 200g, supplied by Liaoning Changsheng Biotechnology Co., Ltd. (license No. SYXK (LIAO) 2013-0009), China. Adaptive breeding for one week before the experiment (temperature: 20～23°C, relative humidity: 50-60%, well-ventilated and suitable illumination). All the studies on animals were in accordance with the Guidelines for the Care and Use of Laboratory animals.

Chemicals and reagents

The herb medicine *Atractylodis rhizoma* purchased from Dalian Branch of Beijing Tongrentang pharmacy (China) were authenticated by Professor Feng Li (TCM Identification department of Liaoning University of Traditional Chinese Medicine), bran frying method reference to pharmacopoeia of China (2015). Atractylenolide I (A I, purity ≥98%), atractylenolide α (Aα, purity ≥98%), atractylenolide β (Aβ, purity ≥98%) and acetaminophen (IS, purity ≥98%) reference substances were purchased from Jiangsu Yongjian Medical Technology Co., Ltd (Jiangsu, China), The chemical structures were shown in fig. 1. HPLC grade methanol, acetonitrile and formic acid were purchased from TEDIA (USA). Ultrapure-grade water was supplied by a Milli-Q ultra-pure water system (Millipore, Bedford, MA, USA).

Preparation of A. rhizoma solution

Same batch crude and processed *A. rhizoma* (50 g each) were crushed into powder and soaked into 600mL of 95% ethanol for 24 h and then percolated at 2mL/min. Ethanol was evaporated to near dryness under reduced pressure to get the residue. Dissolve the residue with purified water before administration and then vortex blending for 10 min. The final concentration of medicinal materials was 2.5 g/mL. The contents of three atractylenolides in crude and processed *A. rhizoma* extracts were determined by a validated HPLC methods established in prior tests, 1.0325 μg/mL (AI), 0.4375μg/mL (Aα) and 0.495μg/mL (Aβ) in the crude one, 1.26μg/mL (AI), 0.5025μg/mL (Aα) and 0.425 μg/mL (Aβ) in the processed one, respectively.

Preparation of standard solution and quality control samples

Mixed standard stock solution was prepared by accurately weighing and dissolving three atractylenolides reference substance in methanol, stored at -20°C and dark place until use. Three atractylenolides in stock solution yielded the following concentrations: AI, 80μg/mL; Aα, 120 μg/mL; Aβ, 100μg/mL. Same method to prepare IS stock solution 132μg/mL, and before use, dilute into 66ng/mL with methanol. Seven calibrators of AI (800, 400, 160, 80, 32, 16 and 6.4ng/mL), Aα (1200, 600, 240, 120, 48, 24 and 9.6 ng/mL) and Aβ (1000, 500, 200, 100, 40, 20 and 8 ng/mL) were prepared by spiking the appropriate amount of the mixed solutions into 200μL blank rat urine. Quality control (QC) samples were prepared at low, medium, and high concentrations of 16, 80, 400ng/mL for AI, 24, 120, 600ng/mL for Aα, and 20, 100, 500ng/mL for Aβ. All the samples were stored at -80°C until analysis.

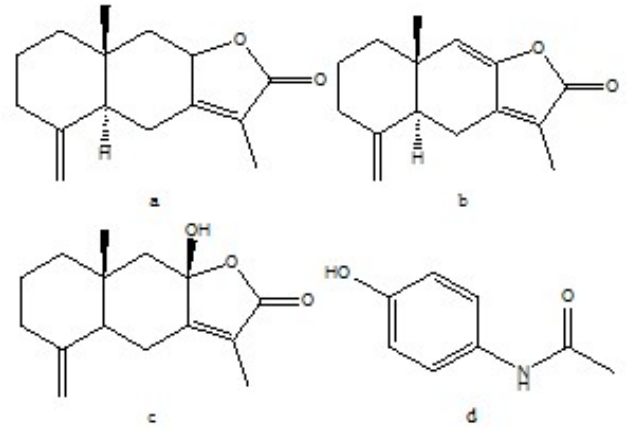

Fig. 1: Chemical structures of atractylenolide I (a), atractylenolide II (b), atractylenolide III (c) and IS (d)

Instrumentation and analytical conditions

An ACQUITY UPLC system (Waters, USA), coupled with a Xevo TQ-S triple quadrupole mass spectrometer (Waters, USA), was used to separate and quantify the analytes in urine under the UPLC-MS/MS conditions obtained from previous work in 10 min.

Separation of the analytes was achieved on an ACQUITY UPLC BEH C18 column (2.1×100 mm, 1.7 μm; waters), with a mobile phase consisted of ACN (A) and 0.1 % aqueous solution of formic acid (B), using a gradient program: 5-10% A at 0-1.5 min; 10-25% A at 1.5-3 min;

25-80% A at 3-6 min; 80% A at 6-8 min; 80-5% A at 8-8.5 min; 5% A at 8.5-10 min. The flow rate was 0.3 mL/min, the column temperature was 40°C and the sample room temperature was 10°C. The analytes were detected by a multiple reaction monitoring (MRM) with an electrospray source in a positive mode. A water Masslynx workstation was used for data acquisition and analysis. The source parameters were set as follows: capillary voltage, 3000 V; desolvation temperature, 400°C; desolvation gas flow, 15 L/min; core gas flow, 2.5 L/min; nebulizer pressure, 7 psi. The MRM analysis was conducted by monitoring the precursor ion to product ion transitions from m/z 233.3→187.2 (AI), m/z 231.2→185.2 (Aα), m/z 249.2→231.2 (Aβ) and m/z 151.9→110.16 (IS). And collision energy was 14 V, 16 V, 8V and 14 V, respectively. The mass spectrogram of analytes and IS were shown in fig. 2.

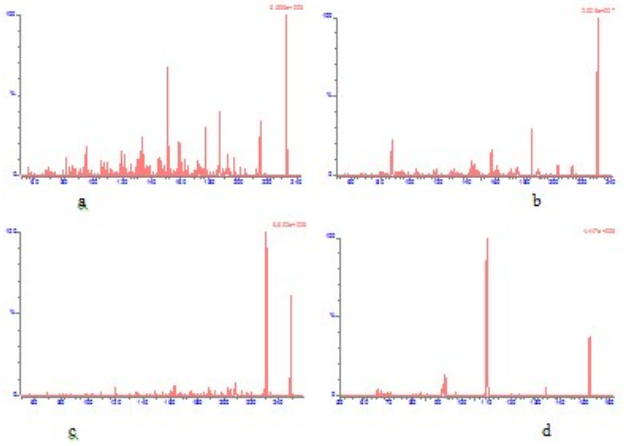

Fig. 2: Mass spectrogram of atractylenolide I (a), atractylenolide II (b), atractylenolide III (c) and IS (d)

Preparation of urine samples

Rats who fasted (free access to water) for 12h were randomly divided into two groups (n=6 per group), then housing in separate metabolic cages and collecting blank urine. Rats were oral administrated with crude and processed *A. rhizoma* extracts at a single dose of 15 mL/Kg, respectively. Urine samples were collected at 0-3, 3-6, 6-9, 9-12, 12-24, 24-36, 36-48, 48-60, 60-72 h post-dosing, and the volume was recorded accurately. The collected urine was centrifuged at 1,000 r/min for 10 min (4°C) and stored at -80°C.

When used, the rats urine was melted at room temperature, while 2mL of them were transferred to the EP tubes, then 100μL IS solution and 900μL methanol were added into every tube. The supernatant of samples were evaporated to dryness at 4°C with nitrogen after vortex blended for 2 min and centrifuged at 1,000 r/min for 5 min (4°C). The resulting extracts were dissolved in 100 μL of methanol, and vortex mixed for 2 min. After they were centrifuged at 12,000 r/min for 15 min (4°C), 2 μL supernatant were injected for analysis, respectively. Finally, the concentration of each analyte was determined.

Method validation

Specificity The specificity of the method was demonstrated by comparing the chromatograms of blank rat urine sample (without IS), blank rat urine sample adding with analytes and IS, and urine samples after an oral administration of crude and processed *A. rhizoma* extracts. All urine samples were prepared and analyzed to ensure the absence of interfering peaks.

Linearity and LLOQ The linearity of the method was assessed by plotting calibration curves of analytes in rat urine at seven concentration levels in triplicate. The LLOQ (lower limit of quantification) was defined as the lowest concentration of the calibration curve of every analyte, while the RSD value was calculated in quintuplicate.

Matrix effect and recovery The matrix effects and recoveries of three analytes were determined at low, medium, and high concentrations in quintuplicate. The matrix effects were measured by comparing the peak area of the analytes in the post-extraction spiked samples with that of the non-processed standard solutions at the same concentrations. The ratios of the analytes peak area in extracted QC samples to those in the post-extraction spiked samples at the same concentration were the extraction recoveries, while the ratios of the calculated concentration to the theoretical concentration were the method recoveries.

The matrix effect and extraction recovery of IS were determined in the same way at the concentration of 66 ng/ml.

Precision and accuracy Intraday precision and accuracy were evaluated by the analysis of low, medium, and high concentrations QC samples in quintuplicate at the same day, while the interday precision and accuracy were measured over three consecutive days. The precision was defined as the relative standard deviation (RSD), while the accuracy was determined by calculating the relative error (RE) of calculated concentration to theoretical concentration.

Stability Short-term (room temperature for 6h) and -80°C freeze-thaw cycles three times stabilities of three analytes in urine samples were investigated by analysing the QC samples at low, medium and high concentrations in quintuplicate. The samples subjected to the different treatments were analyzed, then the results were compared with those of the freshly prepared samples, and the RSD and RE values were calculated.

STATISTICAL ANALYSIS

The excretory kinetic parameters of each rat was calculated by a urine drug analysis model of Drug and

Table 1: The Linear regression analysis of three atractylenolides in rat urine

Compound	Regression equation	r	Linear range (ng/mL)	RSD of LLOQ (%)
A I	y=0.032x+0.383	0.997	6.4 - 800	3.62
A II	y=0.007x+0.123	0.998	9.6 - 1200	3.11
A III	y=0.009x+0.293	0.998	8 - 1000	4.74

Table 2: The results of matrix effect and recovery (n=5, mean ± SD)

	Concentration (ng/mL)	Matrix effect (%)	RSD (%)	Extraction recovery (%)	RSD (%)	Method recovery (%)	RSD (%)
A I	16	102.00±9.94	9.74	80.59±2.86	3.54	78.01±11.6	14.87
	80	101.26±6.91	6.82	94.55±4.65	4.92	99.83±2.88	2.89
	400	105.64±4.75	4.50	86.59±3.94	4.55	95.52±4.08	4.27
A II	24	102.27±4.82	4.71	96.63±6.96	7.20	100.24±11.6	11.57
	120	100.40±7.16	7.13	89.18±4.81	5.39	104.69±6.09	5.82
	600	100.66±6.83	6.79	87.97±4.14	4.71	100.97±6.15	6.09
A III	20	97.60±3.55	3.64	83.89±3.99	4.75	78.01±11.6	14.87
	100	112.99±7.45	6.59	94.76±4.01	4.23	99.83±2.88	2.89
	500	103.82±8.47	8.16	89.11±2.78	3.12	95.52±4.08	4.27
IS	66	111.12±4.11	3.70	94.92±2.99	3.15		

Table 3: The results of precision and accuracy (n=5, mean ± SD)

Compound	Theoretical concentration (ng/mL)	intraday(n=5)			Interday (n=5)		
		Calculated concentration (ng/mL)	RSD (%)	RE (%)	Calculated concentration (ng/mL)	RSD (%)	RE (%)
A I	16	17.25±1.03	5.97	7.83	17.46±0.66	3.76	9.12
	80	84.62±5.43	6.42	5.77	85.48±4.12	4.82	6.84
	400	426.94±3.60	0.84	6.74	424.21±8.53	2.01	6.05
A II	24	24.06±2.78	11.57	0.24	24.61±1.76	7.17	2.54
	120	125.63±7.31	5.82	4.69	126.97±5.02	3.95	5.81
	600	605.81±36.90	6.09	0.97	608.13±21.40	3.51	1.35
A III	20	17.26±0.61	3.53	-13.70	17.20±0.83	4.82	-14.00
	100	99.86±2.88	2.89	-0.14	99.36±3.33	3.35	-0.64
	500	477.75±20.42	4.27	-4.45	481.45±18.86	3.92	-3.71

Table 4: The results of Stability (n=5, mean ± SD)

Compound	Theoretical concentration (ng/ml)	Room temperature for 6h			- 80°C freeze-thaw cycles three times		
		Calculated concentration (ng/ml)	RSD (%)	RE (%)	Calculated concentration (ng/ml)	RSD (%)	RE (%)
A I	16	17.99±0.70	3.92	-2.63	19.01±0.39	2.04	2.86
	80	88.00±2.23	2.53	1.39	89.06±1.31	1.48	2.62
	400	387.39±4.98	1.29	-2.68	408.09±5.26	1.29	2.52
A II	24	25.69±1.53	5.94	4.01	27.93±2.44	8.74	13.06
	120	122.57±1.99	1.62	0.35	119.5±3.34	2.79	-2.16
	600	616.57±17.34	2.81	-0.94	591.73±7.41	1.25	-4.93
A III	20	18.71±1.66	8.88	-12.59	19.30±1.93	9.98	-9.83
	100	90.53±4.99	5.51	-14.28	102.82±3.17	3.08	-2.64
	500	490.67±7.54	1.54	-1.99	507.10±5.58	1.10	1.30

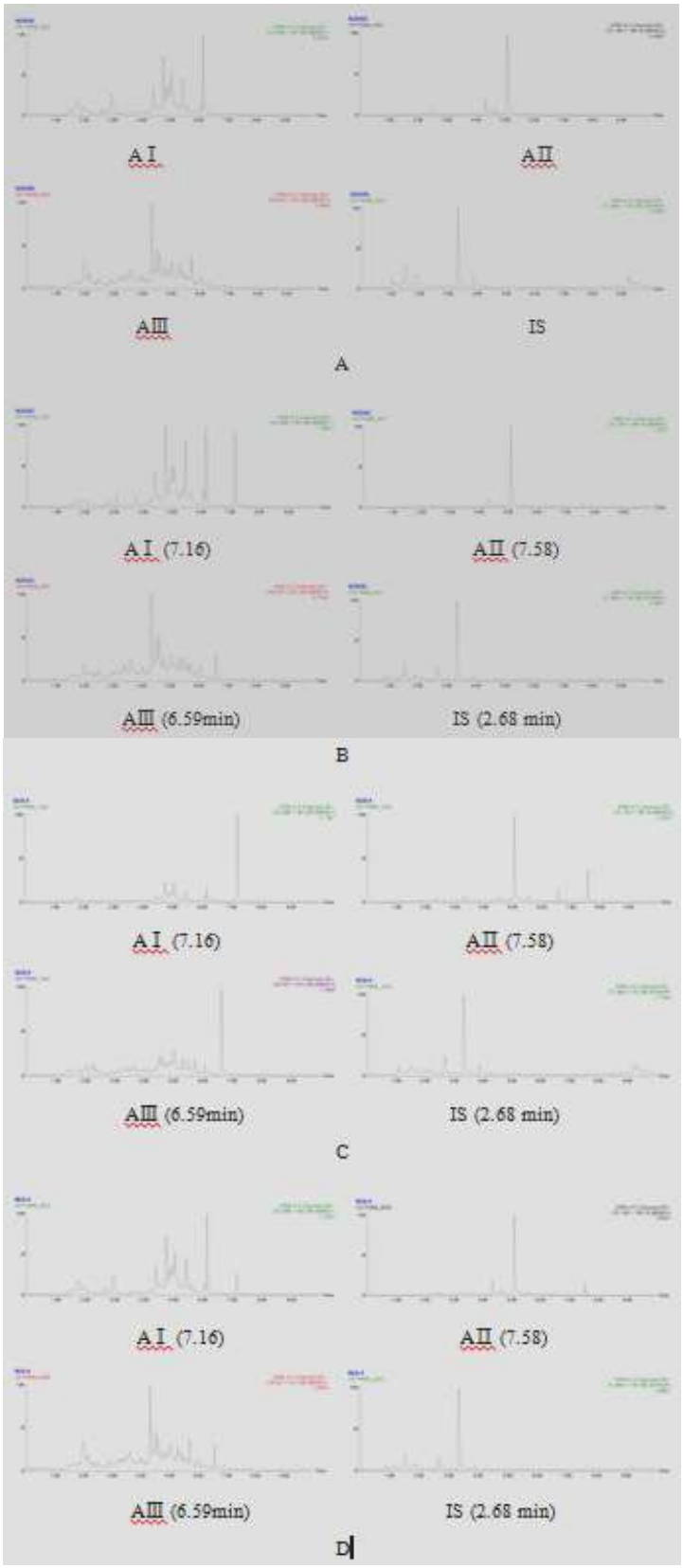

Fig. 3: MRM chromatogram of blank rat urine sample (a), blank rat urine sample adding with analytes and IS (b), rat urine sample after oral administration of crude *Atractylodis rhizoma* (c) and rat urine sample after oral administration of processed *Atractylodis rhizoma* (d)

Statistics (DAS) 3.2.8 software (Chinese Pharmacological Society, Shanghai, China). The final results were expressed as the mean ± standard deviation (SD) and the comparisons between two groups were performed by using the single factor analysis of variance in SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA).

RESULTS

Method validation

Specificity The retention time of IS, AI, Aα and Aβ were 2.68, 7.16, 7.58 and 6.59 min, respectively. The result showed that IS and analytes were well separated and no interferences were detected from endogenous substances or metabolites in urine under the established UPLC-MS/MS condition. The MRM chromatograms of analytes and IS were shown in fig. 3.

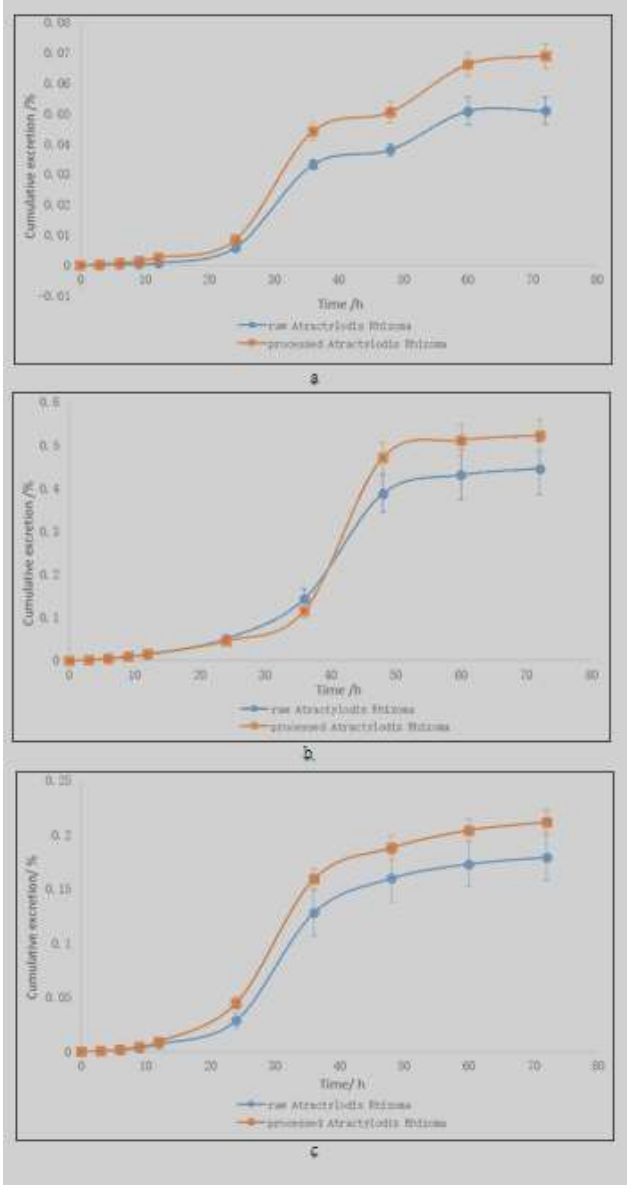

Fig. 4: Recovery cumulative excretion of atractylenolide I (a), atractylenolide II (b) and atractylenolide III (c)

Linearity and LLOQ: The calibration curves of each analyte in urine, with the peak-area ratios of the analytes to the IS (ordinate) plotted against the analyte concentrations (abscissa), was constructed by weighted ($1 \cdot C^{-2}$) least-squares linear regression analysis. All the weighted calibration curves showed good linearity within certain ranges. The regression equations, correlation coefficients, linear ranges, and RSD of LLOQ for all three analytes were shown in table 1.

Matrix effect and recovery: The matrix effects of three atractylenolides and IS varied from 97.6% to 112.99%, and the values of extraction recoveries were in the range from 80.59% to 96.63%. The method recoveries of analytes ranged from 78.01% to 104.69%. The results met to the requirements for quantitative analysis of biological samples. The matrix effect and recovery results of analytes and IS were shown in table 2.

Precision and accuracy: The intraday and interday precisions of three atractylenolides in rats urine changed from 0.84% to 11.57% and 2.01% to 7.17%, while the accuracies ranged from -13.7% to 7.83% and -14% to 9.12 %, respectively. All the results demonstrated that the extraction method was consistent and efficient, and the analysis method was precise and accurate. The precision and accuracy results of three analytes were shown in table 3.

Stability: The stability of three analytes showed no significant sample loss under different treatments. The RSD of two conditions ranged from 1.1% to 9.98%, while the RE changed between -14.28% and 13.06%. The results suggested that the urine samples were stable when these treatments were used. The stability results were shown in table 4.

Excretion study

The assay was applied to the excretory kinetics in rats after oral administration of crude and processed *A. rhizoma* extracts. The main excretory kinetics parameters of three atractylenolides were summarized in table 5 and the recovery cumulative excretion - time profiles of them were illustrated in fig. 4. Compared with the crude *A. Rhizoma* group, the total amount excreted of AI and Aα in the processed group increased significantly, while the recovery cumulative excretion of all three atractylenolides increased.

DISCUSSION

There are little atractylenolides were excreted in rats urine within 24 hours. As shown in fig. 4, Aα and Aβ could reach the plateau at about 48 hours after oral administration of crude and processed *A. rhizoma* extracts, while AI raised markedly at 24-60 hours and reached balance at 60 hours, which may indicated that the

Table 5: Main pharmacokinetic parameters of three atractylenolides compounds (n = 6, mean ± SD)

Compound	$t_{1/2}$ (h)[a]		ke (h^{-1})[b]		Total amount excreted (ng)		Recovery cumulative excretion (%)	
	crude	processed	crude	processed	crude	processed	crude	processed
A I	32.48±8.69	35.05±7.29	0.022±0.005	0.020±0.004	1577.19±137.86	2610.37±152.36**	0.051±0.004	0.069±0.004**
A II	32.34±4.41	33.03±2.64	0.022±0.003	0.021±0.002	5852.53±794.98	7754.87±130.94**	0.446±0.061	0.514±0.009*
A III	44.58±7.21	46.43±6.31	0.016±0.003	0.015±0.002	2660.24±314.64	2700.25±141.79	0.179±0.021	0.212±0.011**

[a] $t_{1/2}$: Biological half-life. [b] ke: Elimination rate constant.
* $P < 0.05$. ** $P < 0.01$, vs. Crude *Atractylodis rhizoma*

absorption of AI *in vivo* was relatively slow. The data in table 5 illustrated that t1/2 of AI and Aα were comparatively consistent, while Aβ were longer than them. The results showed that only a small amount of atractylenolides were excreted from the urine with the prototype, which suggested that the urinary excretion of them was not the major pathway of rats after oral administration of crude and processed *A. Rhizome* extracts at a dose of 37.5g/Kg. It was supposed that the little excretion of atractylenolides might be associated with their absorption and transformation *in vivo*, and perhaps they were excreted in metabolites or other forms.

According to the results, the $t_{1/2}$ and Ke of three atractylenolides had no obvious difference between crude and processed *A. Rhizome*. But compared with crude *A. Rhizome*, the recovery cumulative excretion of processed ones increased significantly (p<0.05, or p<0.01). This might illustrate that stir-frying with wheat bran could promote the excretion of atractylenolides in rat urine.

CONCLUSION

A simple, specific, and rapid UPLC-MS/MS method for the quantification of A I , Aα and Aβ in rats urine was developed for the first time, and successfully applied to a preliminary excretion study of atractylenolides after oral administration of crude and processed *A. rhizoma* extracts at a dose of 37.5g/Kg, respectively. The excretory kinetics parameters $t_{1/2}$ and Ke of three atractylenolides had no significant difference after using Student's t-test, while the recovery cumulative excretion of them in processed *A. rhizoma* were obvious higher than the crude ones (p<0.05,or p<0.01). The results indicated that only a small amount of atractylenolides was excreted in urine with a prototype. Compared with crude *A. rhizome*, stir-frying with wheat bran could increase the excretion of atractylenolides in rats urine. The study of the urinary excretion regularity can provide the evidence for the processing mechanism of *A. rhizoma*, inferring that the excretory change of three atractylenolides may be related to the enhance of tonifying spleen effect in processed *A. rhizoma*.

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