ORIGINAL ARTICLE

Bioassay for total serum bioactivity of *Atractylodes lancea*

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ABSTRACT

The study aimed to establish a bioassay for total bioactivity of *Atractylodes lancea* (AL) in human serum samples. Inhibition of bacterial growth (*Staphylococcus aureus* ATCC 25923) was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The calibration curve (0, 0.39, 0.78, 1.56, 3.13, 2.56, and 50 ng/μl) was linear with correlation coefficients >0.990. The limit of quantification (LOQ) was 1.66 μg/ml using 20-μl serum sample. The developed bioassay method meets the standard of the bioanalytical method for determination of serum bioactivity of AL.

Key words: *Atractylodes lancea*, atractylodin, bioassay, cholangiocarcinoma, pharmacokinetics, *Staphylococcus aureus* ATCC 25923

INTRODUCTION

Atractylodes lancea (AL) is used in China, Japan, and Thailand for treatment of rheumatic diseases, digestive disorders, night blindness, influenza, fever, cold, *etc.*[1-3] These ethnopharmacological uses are supported by the wide range of pharmacological activities of AL in various diseases.[4,5] A series of studies conducted by our group confirms the potential of AL as a chemotherapeutic for cholangiocarcinoma (bile duct cancer).[6-8]

Bioanalysis of AL is challenging due to its complexity and large number of constituents.[9] Gas chromatography, high-performance liquid chromatography, liquid chromatography-mass spectrometry, and ultra-performance liquid chromatography coupled with electrospray ionization LTQ-Orbitrap high-resolution mass spectrometry have been widely employed for the identification and quantitation of the two main bioactive compounds, atractylodin and β-eudesmol.[8,10-15] As several components may contribute to each AL pharmacological activity, it is likely that bioanalysis and investigation of the concentration–time profile (pharmacokinetic study) of a single constituent by these methods may not be correlated well with the overall pharmacodynamic activity of AL. Bioassay which measures total activity of all constituents, however, would better reflect the sum of bioactivity of all components in the herbal extract. The present study aimed to establish a high-throughput bioassay for measurement of the total bioactivity of AL against cholangiocarcinoma cells in human serum samples compared to the high-performance liquid chromatography-ultraviolet (HPLC-UV) method. The pharmacokinetic study of AL was successfully characterized in Thai patients with advanced stage cholangiocarcinoma using the developed method.

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MATERIALS AND METHODS

Chemicals

Atractylodin was provided by Shanghai Run-Biotech Co.,

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Ltd. HPLC-grade reagents were provided by Fisher Scientific. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and other chemicals were provided by Sigma Chemical Co.

Bioassay for measurement of serum ATD

Preparation of bacterial suspension

Biochemical identification of *Staphylococcus aureus* ATCC 25923 strain was performed using the previously described method.[16] Blood agar (Difco Labo.) and LB broth (Difco Lab.) were used to isolate bacterial colonies (37°C, 18–20 h). The colonies were suspended to the turbidity of the 0.5 McFarland standard to achieve the density of 1×10^4 colony-forming unit (CFU)/µl.[17]

Preparation of drug solutions

Atractylodin (ATD) stock solution of 1,000 ng/µl was prepared in dimethyl sulfoxide and two-fold serially diluted with human blank serum (heat-activated) to prepare working solutions (0.39, 0.78, 1.56, 3.13, 2.56, and 50 ng/µl).

Bioassay

Serum samples were heat-activated at 60°C to remove microbial contamination. Serum (20 µl) containing ATD (0, 0.39, 0.78, 1.56, 3.13, 2.56, and 50 ng/µl; triplicate each) was pipetted into each well of the 96-well microtiter plate (Thermo Fisher Scientific Instruments Co., Suzhou, Jiangsu, China) containing bacterial suspension (150 µl, 1×10^4 CFU *per* µl) in LB broth and cultured in an incubator (37°C, 18–24 h).

Assessment of Staphylococcus aureus growth

Each microtiter plate well was added with the MTT solution (10 µl of 5 mg/ml) and left at 25°C for 5 min. Optical density (OD) was read at 570 nm. Bacterial growth (%) was determined as the ratio of the OD of drug-treated wells/OD of control wells. The calibration curve (x-axis: log ATD concentration *vs.* y-axis: % bacteria growth) *vs.* the x-axis (log ATD concentration) was prepared and analyzed by nonlinear regression (CalcuSyn™, Biosoft, Cambridge, UK).

The total serum bioactivity of AL against *S. aureus* was determined from the calibration curve and expressed as ATD equivalent concentration (ATDeq-Sa). The total serum bioactivity of AL against cholangiocarcinoma cells was obtained by multiplying the ATDeq-Sa with the IC_{50} ratio of AL in cholangiocarcinoma and *S. aureus* (multiplying factor) and expressed as ATDeq-cca. The average IC_{50} (50% inhibitory concentration) of the AL extract in various cholangiocarcinoma cell lines is 30 µg/ml.[6] The IC_{50} of AL crude extract for *S. aureus* determined in the present study is 11.42 µg/ml. Therefore, the multiplying factor used to determine the total bioactivity of AL against cholangiocarcinoma cells is 30/11.42 = 2.63.

Assay validation

Calibration curves for serum samples were prepared by spiking with ATD (0.39, 0.78, 1.56, 3.13, 2.56, and 50 ng/µl).

The within-day precision of the assay was evaluated by the analysis of serum samples (20 µl each) spiked with ATD at 0.39, 0.78, 1.56, 3.13, 2.56, and 50 ng/µl (n = 6 each). The day-to-day precision was determined using the same concentration range, but on 6 consecutive days. The coefficient of variation (%CV) was calculated from the ratio of standard deviation (SD) and mean and multiplied by 100.

The accuracy was evaluated by the analysis of six sets of samples spiked with ATD at 0.39, 0.78, 1.56, 3.13, 2.56, and 50 ng/µl (n = 6 each). The difference between spiked concentrations and concentrations added is expressed as % mean deviation (%MDV).

The quantification limit (LOQ) of the assay was determined from the lowest ATD concentration that inhibited bacterial growth.

High-performance liquid chromatography-ultraviolet analysis of plasma ATD

The standard HPLC-UV method was used to determine plasma ATD concentrations.[18] Plasma sample was precipitated with acetonitrile and extracted with dichloromethane. The system consisted of the solvent pump, solvent degasser, autosampler, UV detector at the wavelength of 340 nm (Thermo Fisher Scientific, CA, USA), reversed-phase column (Thermo Hypersil Gold C18, 5 µm), mobile phase (acetonitrile (70%): distilled water (30%), 1.0 ml/min), and Millennium 2000 Chromatograph™ software (Waters Co. Ltd.). All calibration curves (2.5–500 ng/ml) yielded a linear relationship with $r > 0.999$. The %CV of ATD analysis was below 5% at all concentrations (25, 100, and 500 ng/ml). %CV for the intraday and interday assay precision varied between 0.9% to 2.9% and 2.2% to 3.3%, respectively. The MDV values for the intraday and interday assay accuracy varied between 0.2% to 6.1% and 2.9% to 7.0%, respectively. The mean (+SD) recovery of ATD was 76.2%. The LOQ of ATD was 2.5 ng/ml. Plasma samples containing ATD (25, 100, and 500 ng/ml) were found to be stable at −80°C, and three cycles of freeze and thaw procedures.

Application of bioassay for pharmacokinetic study

The pharmacokinetic study of the total serum bioactivity (anticholangiocarcinoma) of AL (ATDeq-cca) was conducted in five Thai patients with advanced stage cholangiocarcinoma (48–62 years of age, 50–65 kg), who received treatment with oral dose of 1,000 mg AL extract (Kao Laoor Co. Ltd., Thailand). Plasma concentrations of ATD in plasma were determined by HPLC-UV. The Ethics Committee of Thammasat University approved the study protocol. Participants gave written informed consents before the study. Venous blood (6 ml) was collected (3 ml into the heparinized

tube and 3 ml into the plain tube) at the following time points: 0 (before dose) and 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, and 6 h after dosing. Plasma (for ATD analysis) and serum (for analysis of the total bioactivity) samples were separated (centrifugation at 2,000×g for 15 min) and stored at −20°C.

RESULTS AND DISCUSSION

Bioassay for determination of total bioactivity of AL in serum samples

All calibration curves (0.39-50 ng/µl ATD) yielded linear relationship with correlation coefficients >0.990 [Figure 1]. The variation of ATDeq-cca assay in serum samples was low, with %CV below 15% at the three concentrations (0.78, 6.50, and 25.00 µg/ml). The assay provided a good intra- and interday precision (%CV: 6.49%–14.63%) and intra- and interday accuracy (MDV: −3.01–+10.55%) [Table 1]. The LOQ of the assay was 1.66 µg/ml using 20-µl serum sample.

Application of the bioassay method for human pharmacokinetic study

The ATDeq-cca concentrations representing total anticholangiocarcinoma activity of AL in serum samples from the five patients are presented in Table 2. The average maximum serum bioactivity of 16.54 ATDeq-cca concentration was achieved at 0.5 h. At the last sampling time point (6 h), ATDeq-cca was not detectable in two patients, while low activity (2.63–9.70 ATDeq-cca) was detected in three patients.

ATD concentrations at various time points in the five patients are presented in Table 3. The average maximum plasma concentration of ATD of 65.90 ng/ml was achieved at 2.5 h. At the last sampling time point (6 h), ATD was not detectable in two patients, while low concentrations (5.20-14.95 ng/ml) were detected in three patients. Comparison of serum bioactivity expressed as ATDeq-cca and plasma ATD concentrations in all patients is presented in Figure 2. It was noted a marked difference in the pharmacokinetic profiles of AL when using ATD and ATDeq-cca as biomarkers for anticholangiocarcinoma.

CONCLUSIONS

A bioassay based on the colorimetric analysis of the total

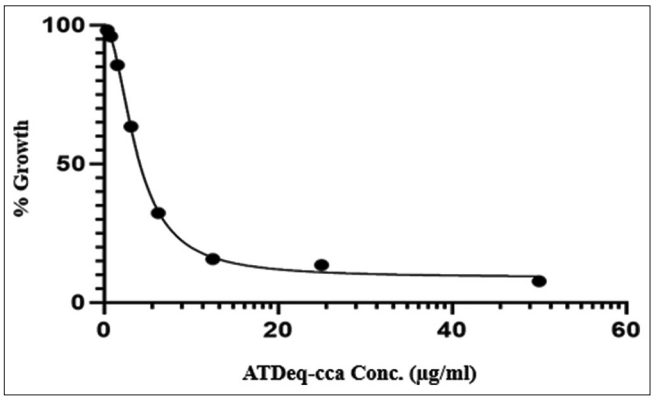

Figure 1: Calibration curve (0.39-50 µg/ml) of the total bioactivity of AL in serum against *Staphylococcus aureus* (expressed as ATDeq-cca)

Table 1: Assay precision and accuracy (percentage DMV) for the total serum bioactivity of *Atractylodes lancea* determined by bioassay (atractylodin equivalent concentration)

Concentration of ATD added (mg/ml)	Precision (% CV)		Percentage DMV	
	Intraassay	Interassay	Intraassay	Interassay
25.00	14.63	8.17	−6.91	−4.15
6.50	9.52	8.33	+6.04	+3.01
0.78	6.49	7.92	+10.55	+9.69

CV: Coefficient of variation, ATDeq-cca: Atractylodin equivalent concentration, DMV: Deviation of mean value

Table 2: Serum total bioactivity (anticholangiocarcinoma activity) of *Atractylodes lancea* extract expressed as atractylodin equivalent concentration

Time (h)	ATDeq-cca (µg/ml)					
	Number 1	Number 2	Number 3	Number 4	Number 5	Mean±SD
0	0	0	0	0	0	0
0.5	16.72	14.93	17.62	16.91	7.88	16.54±4.0
1.0	23.30	8.52	10.04	10.70	5.92	13.14±6.74
1.5	13.93	6.89	11.23	5.65	4.38	9.42±4.01
2.0	10.09	2.39	15.22	7.99	3.94	8.92±5.10
2.5	10.94	5.49	9.28	7.83	6.63	8.38±2.14
3.0	7.31	0	12.43	4.73	4.86	6.12±4.5
4.0	8.73	0	8.65	3.70	3.07	5.27±3.79
5.0	4.10	0	6.65	2.20	4.14	3.24±2.47
6.0	2.68	0	9.70	0	2.69	3.09±3.97

ATDeq-cca: Atractylodin equivalent concentration, SD: Standard deviation

Table 3: Plasma concentrations of atractylodin analyzed by high-performance liquid chromatography-ultraviolet

Time (h)	ATD (ng/ml)					
	Number 1	Number 2	Number 3	Number 4	Number 5	Mean±SD
0	0	0	0	0	0	0
0.5	4.76	10.9	126.91	10.56	13.03	33.23±52.45
1.0	5.57	48.69	147.43	15.91	32.04	49.92±56.9
1.5	7.07	58.90	58.27	23.45	41.44	37.82±22.51
2.0	14.12	70.42	42.20	40.90	61.32	45.79±21.72
2.5	46.04	92.1	48.31	52.68	90.41	65.90±23.26
3.0	38.48	83.56	26.61	70.74	68.48	57.57±23.22
4.0	29.84	56.80	14.82	61.20	40.06	40.54±23.93
5.0	12.21	23.32	7.20	47.33	25.01	23.01±19.15
6.0	0	5.20	0	10.20	14.95	6.07±15.5

ATD: Atractylodin, SD: Standard deviation

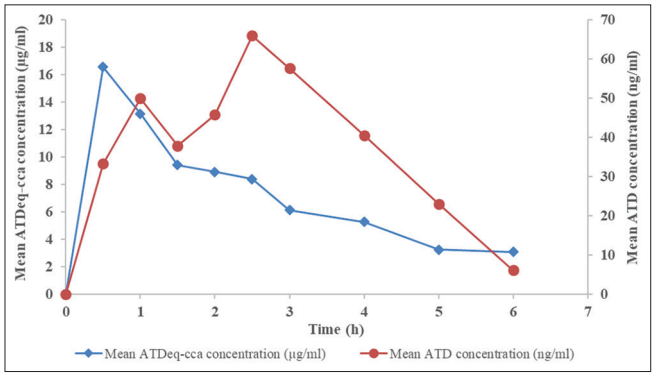

Figure 2: The mean serum AL bioactivity (expressed as ATDeq-cca) and plasma ATD concentrations in five patients with advanced stage cholangiocarcinoma

bioactivity of AL against cholangiocarcinoma in human serum fulfills the criteria of analytical assay performance.[19,20] The assay results reflect the sum of the activity of all identified and unidentified active constituents. The end point of measurement was bacterial growth inhibition assessed by MTT assay, which provides a high-throughput platform for the assay. This would offer a realistic analysis of the pharmacokinetic-pharmacodynamic relationship of AL in patients who receive AL for cholangiocarcinoma treatment and bacterial infections.

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Conflicts of interest

There are no conflicts of interest.

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